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Circular RNA expression profiles of peripheral blood mononuclear cells in rheumatoid arthritis patients

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Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease involving synovial inflammation and joint destruction. Lack of knowledge on RA pathogenesis hinders development of effective treatments. The aim of this study was to examine whether circular RNAs (circRNAs), which are involved in the development of other diseases, may also play a role in RA. Expression profiles of circRNAs were compared between peripheral blood lymphocytes from RA patients and healthy controls. Candidate RA-associated circRNAs were validated using RT-PCR, and interactions with target microRNAs were predicted. Analysis of circRNA microarrays identified 689 circRNAs expressed at significantly different levels between RA patients and controls ($p < 0.05$), of which 95 were up-regulated and 594 down-regulated in RA. Microarray analysis was corroborated by RT-PCR for hsa_circRNAs 103571, 101319, 102034, 103503 and 100257. Potential target miRNAs for hsa_circRNAs were predicted. Several circRNAs in peripheral blood lymphocytes may be differentially expressed in RA (hsa_circRNAs 103571, 101319, 102034, 103503 and 100257). These circRNAs may serve as potential targets for RA diagnosis and treatment.

Key words: Circular RNA, microarray, rheumatoid arthritis, microRNA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease involving synovial inflammation and joint destruction that affects approximately 1% of the general population. It is widely accepted that the pathogenesis of RA is characterized by systemicinflammation and autoimmunity with multiple adjacent cartilage and bone lesions (McInnes and Schett, 2011). RA is a chronic disease with severe complications, whichresults in short- or long-term disability and increased mortality. However, the etiology and pathogenesis of RA remain largely unknown, which hinders efforts to develop therapeutic strategies.

Both genetic and environmental factors are thought to play a role in disease development and disease

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progression. Efforts to identify genetic factors of RA have focused on HLA, PTPN22 and CTLA4, with over 100 genetic risk loci described so far (Wellcome Trust Case Control, 2007). Each risk locus harbors multiple candidate genes and whether any of these genes actually cause the disease is unknown.

Circular RNAs (circRNAs), non-coding RNAs of which more than 25,000 exist in various human cell types (Chen et al., 2015; Jeck et al., 2013), have been implicated in the development of several types of diseases, including atherosclerosis and nervous system disorders (Burd et al., 2010; Chen et al., 1990; You et al., 2015). These RNAs undergo splicing even though they lack a free 3′ or 5′ end (Hentze and Preiss, 2013; Vicens and Westhof, 2014), and they are expressed in certain tissues at certain developmental stages (Memczak et al., 2013; Salzman et al., 2013). Though circRNAs has been measured in tissue, serum, exosomes and other body fluids in several kinds diseases (Kleaveland et al., 2018; Kristensen et al., 2018; Zhou et al., 2018), few studies have examined whether circRNAs may play a role in RNA.

To address this question, the present study used circRNA microarrays to compare expression profiles in peripheral blood lymphocytes between RA patients and healthy controls. The resulting set of differentially expressed circRNAs was analyzed using bioinformatics to predict potential target miRNAs. These results may help identify circRNAs that can serve as RA biomarkers, and their further study may help elucidate pathways driving RA pathogenesis.

MATERIALS AND METHODS

Three RA patients (1 man and 2 women; age range of 50 to 62 years) were recruited from outpatients at the Department of Physical Medicine in the First Affiliated People's Hospital of Xinyang. The patients were diagnosed of RA based on American College of Rheumatology criteria. As controls, three healthy volunteers were recruited (1 man and 2 women; age range of 49 to 63 years).

None of the subjects in this study had diabetes, malignancy or diseases of the kidney or liver, any of which can influence the circRNA expression profile. The study protocol was approved by the Ethics Committee of Xinyang Normal University, and informed consent was obtained from all subjects.

Analysis of circRNA microarrays

Peripheral blood lymphocytes were isolated from RA patients and controls as described (Viecceli et al., 2017). RNA from each sample was subjected to microarray analysis and hybridization according to the manufacturer's protocol (Arraystar, MD, USA). In brief, total RNA was digested with RNaseR (Epicentre, CA, USA) to remove linear RNAs, and the enriched circular RNAs were amplified and transcribed into fluorescent cRNA using a random priming method (Super RNA Labeling Kit, Arraystar). The labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using the NanoDrop ND-1000. Labeled cRNA (1 μg) was fragmented by adding 5 μl 10 × Blocking Agent and 1 μl 25 × Fragmentation Buffer, the mixture was heated at 60°C for 30 min, and finally the labeled cRNA was diluted with 25 μl of 2 × hybridization buffer. Hybridization solution (50 μl) was dispensed into the gasket slide, which was assembled with the circRNA expression microarray slide. Slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven (G2545A). The hybridized arrays were washed, fixed and scanned using the Agilent Scanner G2505C; array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Normalization and subsequent data processing were performed using the R software package. Volcano plot filtering was used to identify circRNAs that differed significantly between patient and control samples. Hierarchical clustering was used to generate circRNA expression profiles for the two groups and to compare them.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was reverse-transcribed into cDNA in reactions containing 1.0 μg of purified total RNA, 4.0 μl of reaction buffer, 0.5 μl of random primers, 0.5 μl of oligo(dT), 2.0 mM dNTP, 0.5 μl of reverse transcriptase and 0.5 μl of RNase inhibitor. Quantitative RT-PCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA), SYBR Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) and the primers shown in Table 1. Three independent samples were analyzed in triplicate for RA patients and controls, with β-actin as internal control. Primer specificity was confirmed by melt curve analysis after the PCRs. Relative expression of circRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Bioinformatics analysis

Interactions between circRNAs and potential target miRNAs were predicted using Arraystar miRNA target prediction software, which is based on TargetScan (www.targetscan.org) and miRanda. This analysis was used to identify the five most likely target miRNAs, whose putative target genes were identified by Targetscan.

Statistical analysis

All experiments were performed and analyzed in triplicate. Results are reported as mean ± standard deviation (SD). Differences between patients and controls were assessed for significance using Student's *t* test, and a threshold of $P < 0.05$ was defined for statistical significance. Data were analyzed using SPSS 23 (IBM, Chicago, IL, USA).

RESULTS

circRNA expression profiles of peripheral blood lymphocytes

Hierarchical clustering was used to generate circRNA expression profiles for RA samples and control samples (Figure 1A), and scatter and volcano plots revealed differences between the circRNA expression profiles

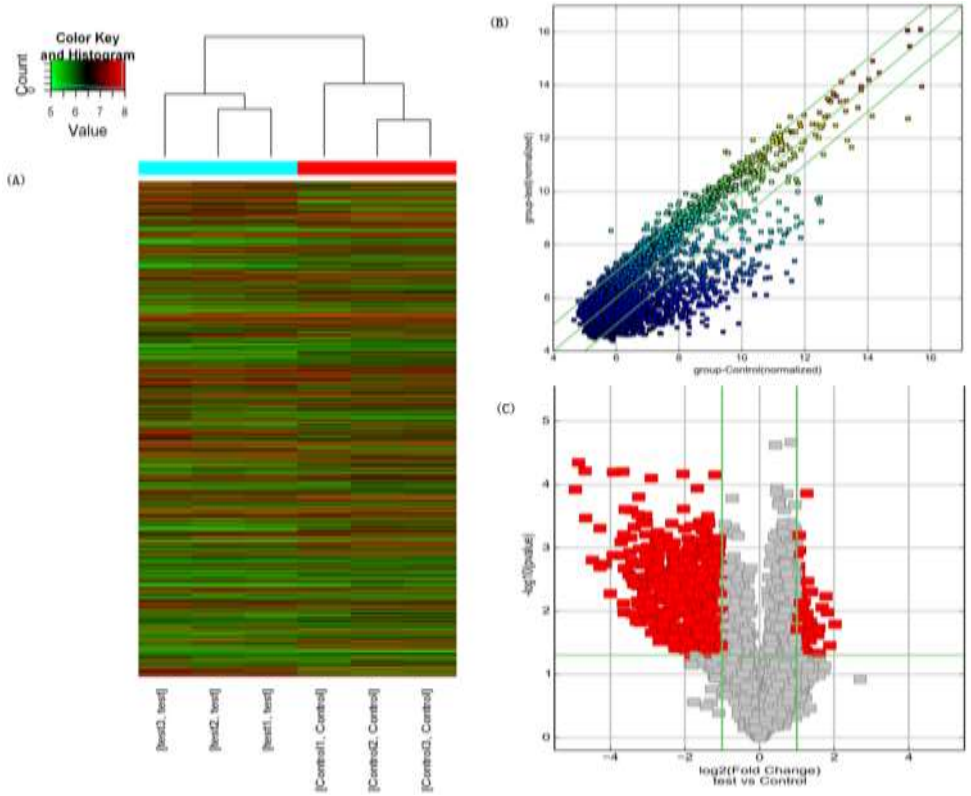

Figure 1. Differential expression of circRNAs in peripheral blood lymphocytes from RA patients and healthy controls. (A) Hierarchical clustering analysis, in which each column represents one sample and each row represents one circRNA. Data were analyzed from three patients and three controls. Red color indicates circRNAs up-regulated in RA; green, circRNAs down-regulated in RA. (B) Scatter plot showing variation in circRNA expression between control samples (normal) and RA samples (test). Values on the *x*- and *y*-axes correspond to the normalized signals (log_2 scaled). Green lines indicate fold-changes; circRNAs above the top green line and below the bottom green line vary more than 2.0-fold between control and RA samples. (C) Volcano plots were constructed based on fold-change values and *p* values. The vertical lines correspond to up- and down-regulation by 2.0-fold between control and RA (test) samples. The horizontal line represents a *p* value. Red points represent circRNAs expressed at significantly different levels between controls and patients.

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

Target	Primer sequence	Product size (bp)
β-actin (Human)	F: 5' GTGGCCGAGGACTTTGATTG3' R: 5' CCTGTAACAACGCATCTCATATT3'	73
hsa_circRNA_103571	F: 5'AACTGACGGTGTTGTTCTTTG3' R: 5'AACTGTTTCTGTTGCAGGTGA3'	136
hsa_circRNA_101319	F: 5'CAGCAACAGTGGCAATGAGA3' R: 5'TTATAGGGAGCTTCCAGCATG3'	120
hsa_circRNA_102034	F: 5'GTCATCTGTATAGTGTATGCCGTTA3' R: 5'CAGCTGGAATGGTGATTTCTT3'	138
hsa_circRNA_103503	F: 5'AGCCTGGAATCACGAAGCACA3' R: 5'TGACGATGACGACGAGACAACA3'	136
hsa_circRNA_100257	F: 5'CCTAAGTCTTATCGAACTGCCA3' R: 5'GATGTGTCTTTGAGGGTGTCTTT3'	160

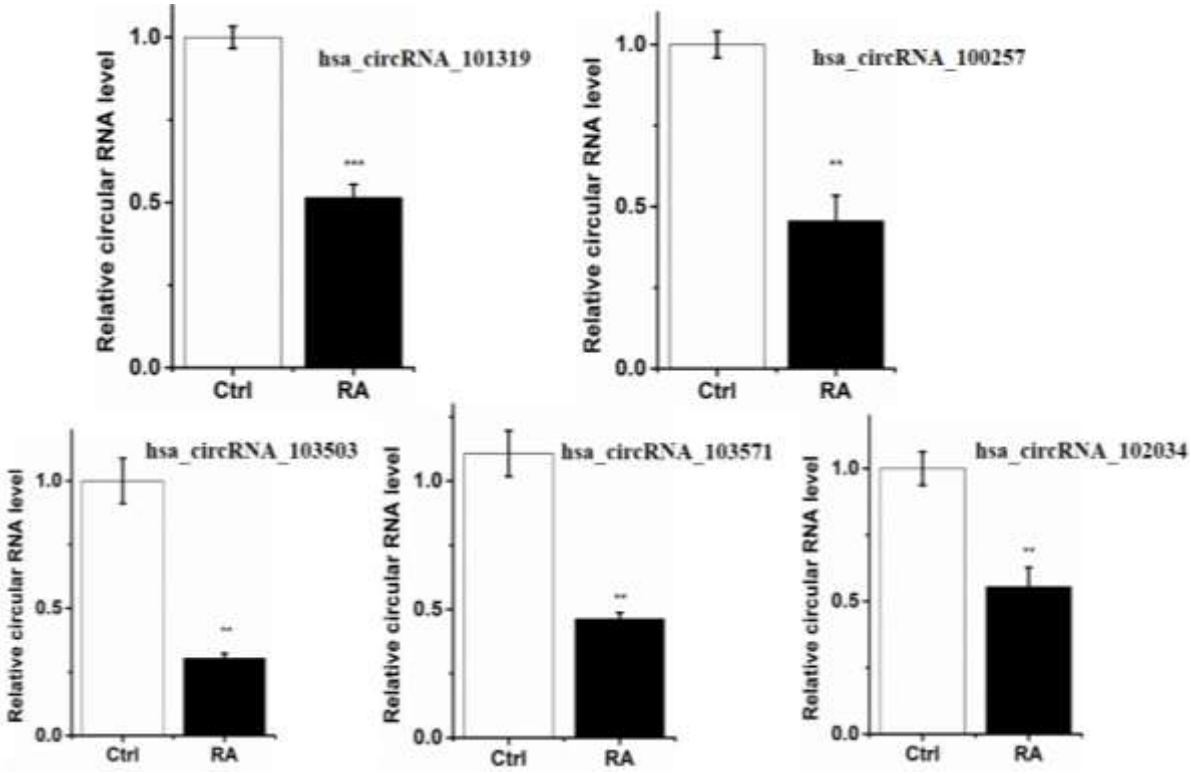

Figure 2. RT-PCR validation of a subset of candidate differentially expressed circRNAs in RA. Data shown are mean ± SD; ** $p < 0.01$, *** $p < 0.05$.

(Figure 1B and C). A total of 689 circRNAs were differentially expressed: 95 were up-regulated in RA, while 594 were down-regulated (Table 1).

Validation of candidate circRNAs using qRT-PCR

The candidate RA-associated hsa_circRNAs 103571, 101319, 102034, 103503 and 100257 were selected and their differential expression was validated using quantitative RT-PCR. The levels of all circRNAs were found to be significantly lower in RA patients (Figure 2), consistent with the microarray data.

Prediction and annotation of circRNA-miRNA networks

Targetscan and miRanda were used to predict miRNAs targeted by the circRNAs differentially expressed in RA. Table 2 shows the top 10 up- and down-regulated circRNAs, together with their predicted miRNA targets. Differentially expressed circRNAs were annotated in terms of their genes (expressing the circRNA or near the genes expressing the circRNA) and miRNA response elements (Figure 3).

DISCUSSION

Many RA studies have focused on the epigenetic regulation of its pathogenesis and potential targets for therapy, including miRNAs and long noncoding RNAs (lncRNAs). In this study, circRNA expression in peripheral blood lymphocytes between RA patients and controls were compared and 689 circRNAs significantly altered in RA patients were identified, out of which, 95 were up-regulated and 594 down-regulated in the disease. However, the occurrence of circRNAs remains largely unknown.

The results show several circRNAs that may be useful as biomarkers or targets in RA. These findings expand the list of diseases in which circRNAs have been implicated (Ghosal et al., 2013; Glazar et al., 2014; Li et al., 2014; Zheng et al., 2016), which include Parkinson's and Alzheimer's diseases (Lin et al., 2016; Lukiw, 2013; Rybak-Wolf et al., 2015), colorectal cancer (Bachmayr-Heyda et al., 2015), oesophageal squamous cell carcinoma (Li et al., 2015a), gastric cancer (Li et al., 2015b), hepatocellular carcinoma (Qin et al., 2016), atherosclerotic vascular disease (Burd et al., 2010), myotonic dystrophy (Ashwal-Fluss et al., 2014) and CD28-dependent CD8(+) T cell ageing (Wang et al., 2015).

Table 2. Characteristics and predicted miRNA targets of the top 10 up- and down-regulated circRNAs in RA*.

P-value	FC (abs)	Level in RA (*vs* healthy)	circRNA	Alias	GeneSymbol	MRE1	MRE2	MRE3	MRE4
0.000119396	30.7312059	down	hsa_circRNA_103503	hsa_circ_0001965	PHC3	hsa-miR-582-3p	hsa-miR-542-3p	hsa-miR-655-3p	hsa-miR-206
4.46066E-05	28.9573651	down	hsa_circRNA_101835	hsa_circ_0005615	NFATC3	hsa-miR-744-5p	hsa-miR-661	hsa-miR-9-5p	hsa-miR-377-5p
6.07889E-05	25.6015442	down	hsa_circRNA_101836	hsa_circ_0000711	NFATC3	hsa-miR-744-5p	hsa-miR-576-3p	hsa-miR-661	hsa-miR-9-5p
0.000338131	25.4155416	down	hsa_circRNA_101550	hsa_circ_0035796	HERC1	hsa-miR-877-3p	hsa-miR-130b-5p	hsa-miR-18a-3p	hsa-miR-26b-3p
0.001560238	22.3442666	down	hsa_circRNA_104052	hsa_circ_0008285	CDYL	hsa-miR-892a	hsa-miR-378a-5p	hsa-miR-887-5p	hsa-miR-22-5p
0.000490694	19.4689185	down	hsa_circRNA_102927	hsa_circ_0058493	RHBDD1	hsa-miR-182-5p	hsa-miR-196a-3p	hsa-miR-28-5p	hsa-miR-330-3p
0.002004659	19.396182	down	hsa_circRNA_103572	hsa_circ_0008351	LRCH3	hsa-miR-29b-2-5p	hsa-miR-544a	hsa-miR-29a-5p	hsa-miR-518c-5p
0.001809156	17.578193	down	hsa_circRNA_101237	hsa_circ_0003489	CDK8	hsa-let-7c-5p	hsa-miR-98-5p	hsa-let-7a-5p	hsa-let-7b-5p
0.005274305	16.0433786	down	hsa_circRNA_101231	hsa_circ_0000467	SKA3	hsa-miR-153-5p	hsa-miR-382-5p	hsa-miR-520g-3p	hsa-miR-549a
0.00130994	15.1884894	down	hsa_circRNA_100913	hsa_circ_0002884	PICALM	hsa-miR-656-5p	hsa-miR-361-3p	hsa-miR-421	hsa-miR-563
0.026636248	2.4575359	up	hsa_circRNA_000046	hsa_circ_0000059	CAP1	hsa-miR-21-3p	hsa-miR-637	hsa-miR-128-2-5p	hsa-miR-296-5p
0.000137307	2.410555	up	hsa_circRNA_000442	hsa_circ_0001625	BACH2	hsa-miR-580-5p	hsa-miR-139-3p	hsa-miR-877-3p	hsa-miR-603
0.016132485	4.0707993	up	hsa_circRNA_000684	hsa_circ_0001013	KIAA1841	hsa-miR-30d-3p	hsa-miR-185-5p	hsa-miR-30e-3p	hsa-let-7b-5p
0.017315747	2.0142591	up	hsa_circRNA_000776	hsa_circ_0001929	XLOC	hsa-miR-128-1-5p	hsa-miR-128-2-5p	hsa-miR-146a-3p	hsa-miR-93-3p
0.0463824	2.0501922	up	hsa_circRNA_001012	hsa_circ_0000254	RRP12	hsa-miR-125b-5p	hsa-miR-125a-5p	hsa-miR-184	hsa-miR-1224-5p
0.004958353	2.6277342	up	hsa_circRNA_001241	hsa_circ_0000508	CUL4A	hsa-miR-342-3p	hsa-miR-449b-3p	hsa-miR-377-3p	hsa-miR-432-3p
0.002992558	2.0532735	up	hsa_circRNA_001754	hsa_circ_0000559	FOXN3	hsa-miR-136-5p	hsa-miR-877-3p	hsa-miR-421	hsa-miR-153-3p
0.015774197	2.1293544	up	hsa_circRNA_001820	hsa_circ_0000728	MC1R	hsa-miR-1296-5p	hsa-miR-770-5p	hsa-miR-384	
0.023862644	2.9435674	up	hsa_circRNA_002039	hsa_circ_0001748	CHCHD3	hsa-miR-624-5p	hsa-miR-28-5p	hsa-miR-511-3p	hsa-miR-545-5p
0.022712291	2.0650012	up	hsa_circRNA_002042	hsa_circ_0000204	WDR37	hsa-miR-1-3p	hsa-miR-22-5p	hsa-miR-656-3p	hsa-miR-302c-5p

FC (abs), fold-change; MRE, miRNA response element; circRNAs are sorted by their fold-change. MREs were predicted using miRNA target prediction software (Arraystar).

How circRNAs function in health and disease is unclear. Evidence is growing that they act as molecular sponges to "mop up" target miRNAs and thereby regulate the ability of miRNAs to inhibit gene expression. For example, the circRNA ciRS-7 contains miRNA-7 binding sites, which allow it to bind miRNA-7 and regulate gene expression in Parkinson's and Alzheimer's diseases (Lin et al., 2016; Lukiw, 2013; Rybak-Wolf et al., 2015). Some of the differentially expressed circRNAs identified in the present study are predicted to bind miRNA targets that have previously been linked to RA. Several studies (Churov et al., 2015; Pauley et al., 2008) have reported up-regulation of hsa-miR-16-5p in peripheral blood mononuclear cells, plasma and synovial fluid of RA patients; miR-16 targets the 3′-UTR of tumor necrosis factor-α, a key pro-inflammatory mediator in RA (Ouboussad et al., 2017; Sujitha and Rasool, 2017). The miRNAs miR-24-3p and miR125a-5p stimulate production of chemokine and inflammatory cytokines and may be helpful in RA diagnosis (Hong et al., 2017; Lai et al., 2017). Many of the predicted miRNA binding sites in the circRNAs identified in the present study are likely to be functional (Hansen et al., 2013; Liu et al., 2017; Migita et al., 2017; Zhang et al., 2017). Future studies should validate the miRNA binding predictions here and exploit these circRNA-miRNA interactions to elucidate the pathogenesis of RA.

CircRNAs can be categorized into three types: exonic, intronic and exonic-intronic. Exonic circRNAs are large molecules comprising exons and are considered by-products of exon skipping, either in pre-messenger RNA (mRNA)

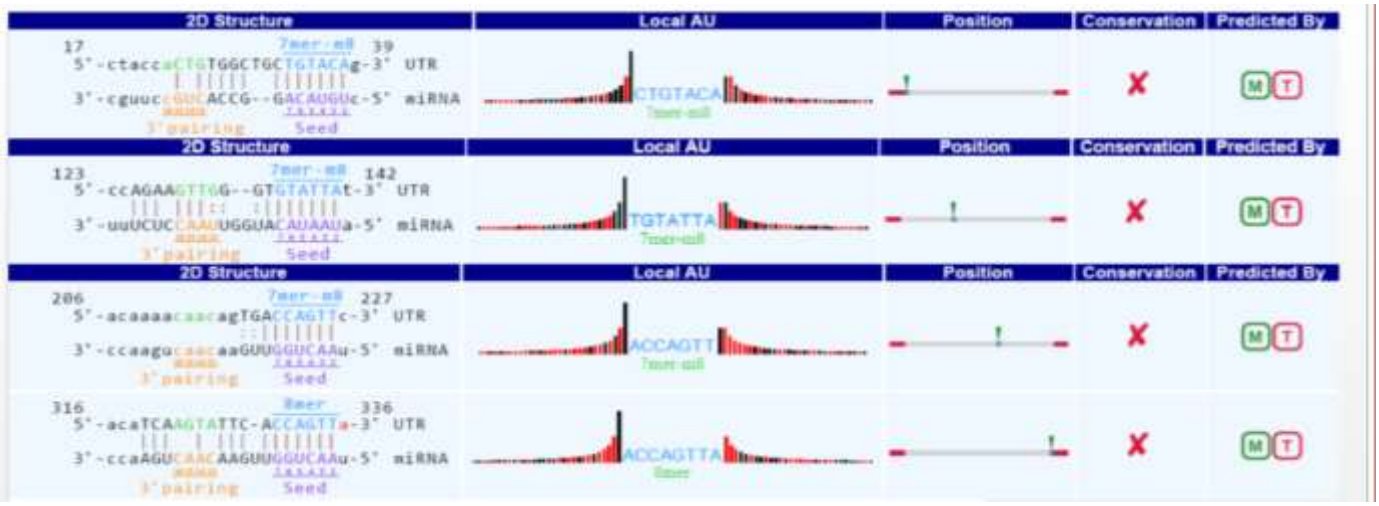

Figure 3. Annotation of predicted circRNA−miRNA interactions. Example of annotation results showing complementarity of hsa_circRNA_103503 with hsa-miR-582-3p, hsa-miR-542-3p and hsa-miR-655-3p.

splicing or in mature mRNA re-splicing (Chen et al., 2016; Wang, 2015). Intronic circRNAs are produced by joining two or more introns, which is rare in eukaryotic cells. Exonic-intronic circRNAs are enriched at transcription sites and may promote transcription of the corresponding parental mRNAs. Recent studies have shown that many exonic transcripts can form circRNAs through non-linear reverse splicing or gene rearrangement. Both exonic and intronic circRNAs may help regulate gene expression (Ebbesen et al., 2016; Li et al., 2015c). In general, circRNAs are more highly conserved and stable than miRNAs and lncRNAs, (Hansen et al., 2013) which makes circRNAs particularly attractive as biomarkers and therapeutic targets (Li et al., 2015b) The present work identifies several circRNAs differentially expressed in RA, and these molecules may help drive the disease by binding miRNAs to regulate gene expression. Future work should examine circRNA-miRNA interactions and the genes affected in order to gain insights into RA pathogenesis as well as explore potential diagnostic tools and therapeutic targets.

CONFLICTS OF INTEREST

The authors have not declared any conflict of interests.

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ABBREVIATIONS

RNA, Ribonucleic acid; RA, rheumatoid arthritis; RT-PCR, reverse transcription-polymerase chain reaction; miRNA, microRNA; lncRNAs, long noncoding RNAs.

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