Identification of circulating miR-22-3p and let-7a-5p as novel diagnostic biomarkers for rheumatoid arthritis

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Abstract

Objective

Early and correct diagnosis would be beneficial for outcomes of rheumatoid arthritis (RA), but there are some limitations in current diagnostic tools. In this study, we aimed to evaluate the diagnostic value of circulating miR-22-3p and let-7a-5p in RA.

Methods

Seventy-six RA patients, 30 systemic lupus erythematosus patients, 32 Sjögren's syndrome patients and 36 healthy donors recruited at the First Affiliated Hospital of Fujian Medical University (China) were included in this study. Circulating miR-22-3p and let-7a-5p in plasma were measured using reverse transcriptase quantitative PCR and serum cytokines were detected by cytometric bead array. The participants' clinical materials were also collected. Receiver operating characteristic curve analysis and correlation analysis were performed to assess the potential value of circulating miRNAs in RA.

Results

Circulating miR-22-3p and let-7a-5p are significantly increased in RA patients and able to distinguish RA patients from other populations. Circulating let-7a-5p has been shown to improve the diagnostic ability of current laboratory indicators anti-cyclic citrullinated peptide antibodies and rheumatoid factor. Moreover, the discriminatory capacity of both circulating miRNAs contribute to complement the diagnosis for seronegative RA.
Meanwhile, correlation analysis reveals that circulating miR-22-3p positively correlates with haemoglobin, serum bilirubin, albumin and IL-17 but negatively correlates with mean platelet volume as well as let-7a-5p.

Conclusion

The increased circulating miR-22-3p and let-7a-5p levels in RA patients, especially in seronegative RA patients, may provide potential promising diagnostic biomarkers for RA in clinical practice.

Key words
rheumatoid arthritis, circulating miRNAs, seronegative RA, diagnostic value

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, which can cause persistent synovitis, joint destruction as well as disability (1, 2). The disorder is most typical in women and occurs at any age. It affects about 0.5 ~ 1.0% of the population worldwide (3) and 0.28% of population in China (4). Despite numerous studies, the aetiology of RA has not been completely elucidated. However, evidences point that genetics, epigenetics, environment, sex, infection and microbiota are involved in the initiation and progression of RA (5, 6). Currently, several available drugs including disease-modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, biological agents and glucocorticoids cannot yet cure RA completely but only control inflammation and delay progression (7). For this reason, timely diagnosis and appropriate treatment are critical for improving the clinical outcome and reducing irreversible joint damage which cause disability (8, 9).

The classification criteria of RA used clinically so far is based on the 2010 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) classification criteria (10). Although the 2010 ACR criteria highlights the early diagnosis of RA compared to the former ACR criteria proposed in 1987 (11), the misdiagnosis and missed diagnosis of RA still occur from time to time. The specific biomarkers for detection of RA provided in the 2010 ACR criteria are anti-cyclic citrullinated peptide antibodies (anti-CCP) and rheumatoid factor (RF), which only show a modest discriminating capability (12, 13). Moreover, when anti-CCP and RF are both negative, more than 10 joints need to be affected in order to be considered as RA according to the 2010 ACR criteria. Therefore, there is an urgent need to develop novel diagnostic tools to improve the diagnostic sensitivity and accuracy of anti-CCP and RF in RA patients, especially in seronegative individuals.

MicroRNAs (miRNAs) are endogenous small (~22 nucleotides) noncoding RNAs known to mediate post transcriptional repression of mRNA targets in diverse species (14). Dysregulation of miRNAs has been described in various diseases, including tumours, autoimmune diseases as well as RA (15-17). Many miRNAs discovered in several cells, tissues and body fluids have been confirmed that are involved in the pathogenesis of RA (18). In our previous study, we demonstrated miR-22-3p promoted fibroblast-like synoviocyte (FLS) proliferation and interleukin (IL)-6 production through targeting Cyr61 (19). Altered expression of let-7a-5p in RA has been found in our unpublished observations or other reports (20, 21). However, the diagnostic value of miR-22-3p and let-7a-5p in RA have been still less investigated. Since several studies have found that miRNAs are stably present in peripheral blood, circulating miRNAs are becoming a novel class of non-invasive biomarkers for prediction, diagnosis and prognosis in various diseases (22-24). This inspired us to ask questions of whether and which circulating miRNAs have an opportunity to become a valuable complement or replacement to RF and anti-CCP in diagnosis. Therefore, we conducted this study to explore the diagnostic value of circulating miR-22-3p and let-7a-5p in RA based on our previous works.

In the present study, we verify that the elevated circulating miR-22-3p and let-7a-5p are observed in patients with RA but not in healthy donors (HD) or patients with systemic lupus erythematosus (SLE) and Sjögren syndrome (SS). Moreover, we evaluate the diagnostic value of both circulating miRNAs in seropositive and seronegative RA patients. To better understand the potential roles of these miRNAs in RA, we also explore the relationship between both circulating miRNAs and the clinical parameters as well as serum cytokines. In summary, these findings support that circulating miR-22-3p and let-7a-5p may be potential biomarkers in the diagnosis of RA.

Materials and methods

Study population and plasma samples

Serum and plasma samples from 76 RA patients, 30 SLE patients, 32 SS patients and 36 healthy donors were obtained between January and October

2020 from the First Affiliated Hospital of Fujian Medical University, Fuzhou, China. As for plasma samples, the peripheral blood samples were collected in EDTA-anticoagulated vacutainer tubes and then centrifuged (3,000 rpm) at 4°C for 10 min to separate plasma followed by RNA extraction immediately.

Patients were included in this study only if RA (2010 ACR/EULAR) (10), SLE (2019 EULAR/ACR) (25) and SS (2012 ACR) (26) patients fulfilled their classification criteria, respectively. All participants who had history of cardiovascular disease, endocrine disease, hepatic disease, renal disease and any other chronic inflammatory diseases were excluded. The study was approved by the local research ethics committee.

Laboratory parameters of all patients and healthy donors were measured and analysed at the local laboratory department. Full informed consent for data collection was obtained from all participants.

RNA isolation and reverse transcriptase quantitative PCR

For miRNA extraction, RNA from freshly human plasma samples was isolated using an miRcute Serum/Plasma miRNA Isolation Kit (Tiangen Biotech, Beijing, China) according to the manufacturers' instructions. Briefly, 200 µl of plasma samples were mixed with 900 µl of lysis solution and vortexed for 30 s followed by the addition of synthetic cel-miR-39-3p standard RNA (1 pmol) purchased from RiboBio (RiboBio Inc, Guangzhou, China). After a series of phenol-chloroform extractions and ethanol precipitation, adsorption columns and collection pipes were used to purify the RNA. Next, columns were washed with the deproteinised solution and the rinsing solution followed by the final elution of RNA performed with 20 µl of RNase-free water.

For reverse transcriptase quantitative PCR (RT-qPCR), the miRNA-specific stem-loop reverse-transcription primers and RT-qPCR primers were designed using the miRNA primer design tool miRNA Design (Vazyme, Nanjing, China). Reverse transcription was performed using the miRNA 1st Strand cDNA Synthesis Kit (Vazyme) in accordance with the manufacturer's protocol. The RT-qPCR reactions were executed using miRNA Universal SYBR qPCR Master Mix (Vazyme) and carried out on a QuantStudio DX (Applied Biosystems Inc., Foster City, USA) according to manufacturer's protocol as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, and at 60°C for 30 s. The threshold cycles (Ct) for the target miRNAs and the synthetic cel-miR-39-3p standard RNA were used to determine their relative expression. The calculation was analysed with the $2^{-\Delta\Delta(Ct)}$ method, where ΔΔCt = [Ct (targeted) – Ct (cel-miR-39-3p)] expt/[Ct (targeted) – Ct (cel-miR-39-3p)] control. Data were analyzed with QuantStudio Real-Time PCR software (Applied Biosystems Inc.). All primers sequences mentioned above are listed in Supplementary Table S1.

Diagnostic value analysis of miRNAs for RA

Receiver operating characteristic (ROC) curve analysis was implemented for target miRNAs and the available clinical indicators with the statistical software MedCalc v. 19.5.1 (MedCalc Inc., Mariakerke, Belgium) to determine the diagnostic value. ROC curves were plotted according to the different cut-off levels calculated by different Youden index. The highest Youdens Index (Youdens index = sensitivity + specificity - 1) was calculated to identify the optimal diagnostic cut-off values, which were used to dichotomised patients. In addition, sensitivity, specificity, 95% Confidence Interval (CI) and the area under the curve (AUC) were also provided by MedCalc.

For combined ROC analysis, the potential predictive factors of RA were identified in univariable logistic regression analysis and significant factors were included in a multivariable logistic reression analysis for controlling the possible effects of confounders using the statistical software SPSS 20.0 (SPSS Inc., Chicago, USA). Then the binary logistic regression was applied to calculate the combined predicted probability based on various combinations of independent variables. Subsequently, the combined ROC analysis was performed in order to find the best performing model using MedCalc Software (MedCalc Inc.). The diagnostic performances, expressed as AUCs, of various diagnostic models were assessed with z-test proposed by DeLong *et al*. (27).

Analyses of clinical parameters

Clinical data were collected from the laboratory system and medical records. Serum anti-CCP level was measured using a commercial ELISA kit (EUROIMMUN, Lübeck, Germany). Serum rheumatoid factor (RF) and C-reactive protein (CRP) were detected by immunoturbidimetric assay (Dade Behring, Marburg, Germany). Erythrocyte sedimentation rate (ESR) was determined using Westergren's method. Biochemical parameters including ALT, AST, TBIL and so forth were analysed by a Siemens ADVIA 2400 analyser while blood routine indexes including RBC, WBC, Hb and so forth were tested using a Siemens ADVIA 2120i analyser (Siemens Healthcare Diagnostics, Erlangen, Germany). The disease activity score assessing 28 joints (DAS28) was evaluated by a rheumatologist using the formula:

$DAS28 = 0.56 \times \sqrt{(TJC28)} + 0.28 \times \sqrt{(SJC28)} + 0.70 \times \ln(ESR) + 0.014 \times VAS$

(range 0–100 mm, 0=inactive disease), TJC28: number of tender joints, SJC28: number of swollen joints, VAS: visual analogue scale.

Detection of cytokines

Cytometric bead array (CBA) was used to quantify multiple human serum cytokines simultaneously including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-17, IFN-γ, IFN-α and TNF-α following the manufacturer's instructions. All reagents were procured from Qingdao RAISECARE Company (RAISECARE Biotechnology Co., Ltd. Qingdao, China) and flow cytometry was conducted on Navios cytometer and analysed using FlowJo software.

Statistical analysis

All statistical differences analysis and correlation analysis were performed using GraphPad Prism 8 statistical software (GraphPad Software Inc., San Diego, USA). Differences between two

Table I. Clinical characteristics of volunteers with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS) patients and healthy donors (HD).

Characteristics	RA	SLE	SS	HD
Number	76	30	32	36
Age (years)	54.36 ± 13.02	39.50 ± 13.44	50.22 ± 14.26	49.33 ± 9.62
Sex (M/F)	15/61	6/24	4/28	8/28
RF (IU/ml)	56 (22.25, 193.25)	-	-	-
anti-CCP (RU/ml)	166.3 (8.71, 200)	-	-	-
ESR (mm/h)	21.5 (12, 36.75)	-	-	-
CRP (mg/l)	5 (2.515, 13.425)	-	-	-
DAS28	4.25 ± 1.30	-	-	-
miR-22-3p relative expression	6.29 (4.20, 9.30)	3.27 (2.22, 4.77)	3.49 (2.55, 5.62)	3.26 (0.95, 4.56)
let-7a-5p relative expression	3.05 (1.66, 6.15)	2.00 (1.04, 3.26)	1.39 (1.09, 1.89)	1.14 (0.65, 1.87)

All values are represented as the mean ± standard deviation or the median (interquartile range) depending on whether the parameters follow a normal distribution.

groups were utilised by the Mann-Whitney U-test to compare quantitative variables. For comparisons among multiple groups, Kruskal-Wallis test followed by Dunn's test was applied. The relationships between clinical parameters and miRNAs were verified using Pearson correlation. All values were represented as the mean ± standard deviation or the median (interquartile range) depending on whether the parameters follow a normal distribution.

All tests were two-tailed, and a p-value <0.05 was considered statistically significant.

Results

Circulating miR-22-3p and let-7a-5p levels are elevated in plasma of RA patients

We first assessed the expression levels of circulating miR-22-3p and let-7a-5p in patients with RA or other types of autoimmune diseases and healthy donors. As shown in Figure 1, levels of miR-22-3p were significantly higher in the plasma of patients with RA in comparison with patients with SLE (p<0.0001), SS (p<0.0001) or HD (p<0.0001). Circulating let-7a-5p was found at significantly higher levels in the plasma of patients with RA than in patients with SS (p=0.0002) and HD (p<0.0001) while it was marginally significantly higher than those of patients with SLE (p=0.0348). The other clinical characteristics of all participants are summarised in Table I. Moreover, further analysis demonstrated that the levels of circulating miR-22-3p and let-7a-5p were not associated with age or gender (data not shown).

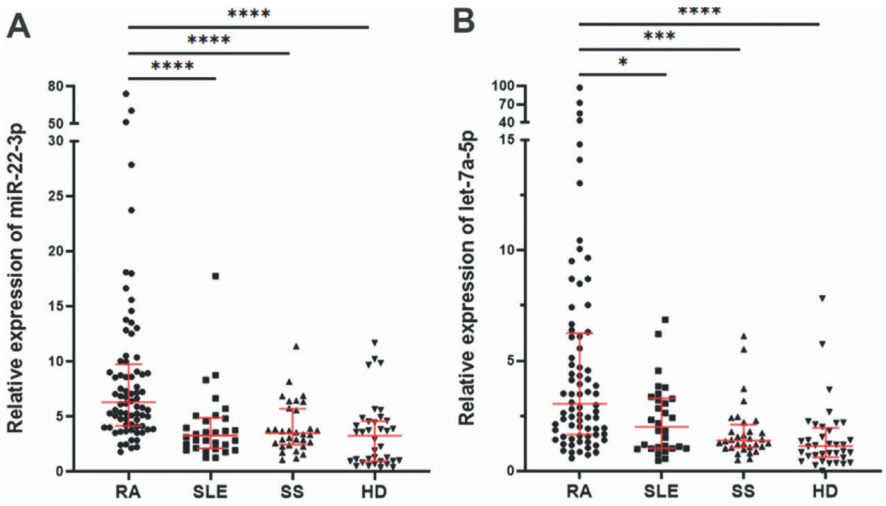

Fig. 1. Increased circulating miR-22-3p and let-7a-5p levels in the plasma of RA patients. The levels of circulating miR-22-3p (**A**) and let-7a-5p (**B**) in plasma of 76 RA patients were higher than that in 36 HD and 30 SLE or 32 SS patients. The p-value derived using Mann-Whitney U-test followed by Dunn's post-test for multiple comparisons is indicated (*p<0.05; ***p<0.001; ****p<0.0001).
RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; HD, healthy donors.

The diagnostic value of circulating miR-22-3p and let-7a-5p in RA

Timely diagnosis and treatment of RA are critical for reducing disability and improving survival quality. Thus, we first examined the diagnostic value of circulating miR-22-3p and let-7a-5p for RA. As presented in Figure 2, ROC curve analysis was performed to evaluate the performance of an individual miRNA (miR-22-3p or let-7a-5p) or combination index of miR-22-3p and let-7a-5p (combined miR-22-3p/let-7a-5p) in differential diagnosis between RA patients and other populations including SLE and SS patients or HD. Detailed results of ROC curves analysis were provided in Supplementary Table S2. In order to further assess the diagnosis value among all indicators, we then compared AUCs using z-test. The comparative results are summarised in Table II.

In the RA *versus* the HD subgroup, our above analysis revealed that miR-22-3p, let-7a-5p or combined miR-22-3p/let-7a-5p had larger AUCs (AUCs >0.8) compared with other subgroups, which indicated a good discriminative capacity of miR-22-3p and let-7a-5p between RA and HD. The combined ROC curve showed miR-22-3p combined with let-7a-5p for the diagnosis were better than the individual miR-22-3p (p=0.0448) but no better than the individual let-7a-5p, which implied let-7a-5p had more contribution to RA diagnosis compared with miR-22-3p in RA *vs*. HD subset.

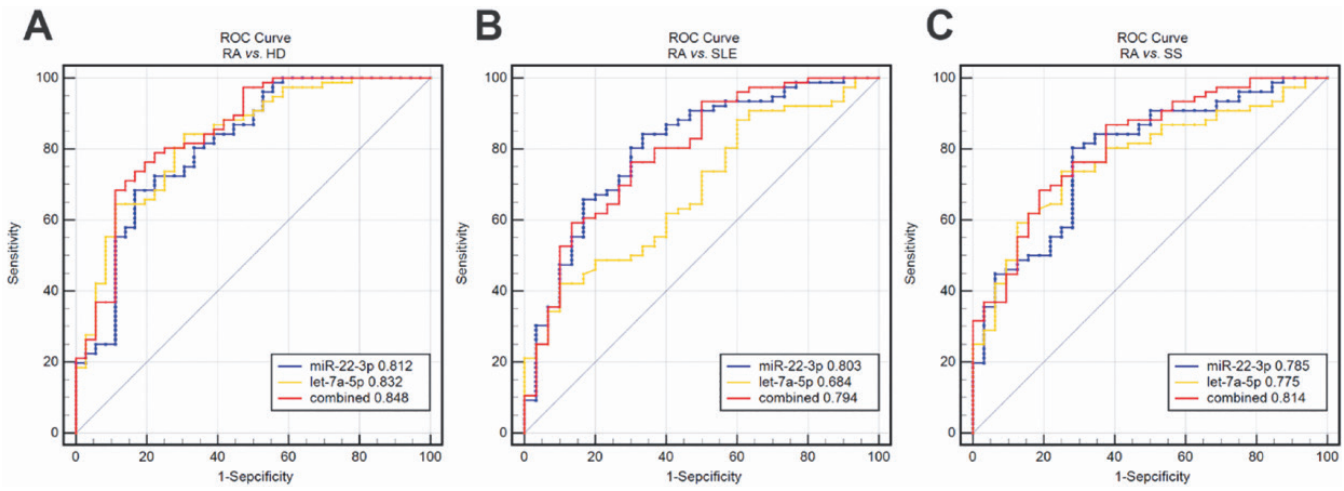

Fig. 2. ROC curves of circulating miR-22-3p and let-7a-5p for RA diagnosis. ROC analysis was performed to evaluate the performance of circulating miR-22-3p, let-7a-5p and miR-22-3p combined with let-7a-5p (combined) in distinguishing RA from HD (**A**), SLE (**B**) and SS (**C**).
Numbers represent the area under the curve.
ROC: operating characteristic curve; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SS. Sjögren's syndrome; HD: healthy donors.

Table II. Comparison of the diagnostic ability among miR-22-3p, let-7a-5p and combined miR-22-3p/let-7a-5p in different subgroups by evaluating the area under curve (AUC) using z-tests (numbers represent *p*-values).

Indicators	AUC		miR-22-3p			let-7a-5p			combined miR-22-3p/let-7a-5p		
		Subgroups	RA *vs.* HD	RA *vs.* SLE	RA *vs.* SS	RA *vs.* HD	RA *vs.* SLE	RA *vs.* SS	RA *vs.* HD	RA *vs.* SLE	RA *vs.* SS
miR-22-3p	0.812	RA *vs.* HD	-								
	0.803	RA *vs.* SLE	0.5929	-							
	0.785	RA *vs.* SS	0.3804	0.7266	-						
let-7a-5p	0.832	RA *vs.* HD	0.5846	-	-	-					
	0.684	RA *vs.* SLE	-	0.0064**	-	0.0073**	-				
	0.775	RA *vs.* SS	-	-	0.8365	0.1922	0.0817	-			
combined	0.848	RA *vs.* HD	0.0448*	-	-	0.4646	-	-	-		
miR-22-3p/	0.794	RA *vs.* SLE	-	0.4241	-	-	0.0019**	-	0.2187	-	
let-7a-5p	0.814	RA *vs.* SS	-	-	0.2251	-	-	0.1812	0.3393	0.7826	-

In comparison to let-7a-5p (AUC= 0.684), miR-22-3p (AUC=0.803) showed better diagnostic discrimination value in subgroup RA *vs.* SLE (p=0.0064) while no significant differences were observed compared with combined miR-22-3p/let-7a-5p (p=0.4241). The results demonstrated miR-22-3p had a good performance in distinguishing RA and SLE. However, in the RA *versus* the SS subgroup, the diagnostic abilities of all indicators were not prominent compared with other subgroups.

Both circulating miR-22-3p and let-7a-5p are not correlated with the clinical parameters of RA

RF, anti-CCP, ESR and CRP are the foremost laboratory indicators for the diagnosis of RA, which have been listed in the ACR/EULAR 2010 classification criteria for evaluating RA progression and disease activity. Therefore, the association of circulating miR-22-3p and let-7a-5p with these laboratory indicators were explored.

First, the levels of RF and anti-CCP were categorised into normal (negative, within the normal reference range) and increased (positive, above the upper limit of normal reference range) according to their respective laboratory reference ranges. Then RA patients were divided into subgroups as follows: RA patients with increased RF (>20 IU/mL) and increased anti-CCP (>5 RU/mL), RA patients with increased RF and normal anti-CCP, RA patients with normal RF and increased anti-CCP, RA patients with normal RF and normal anti-CCP. Using the similar classification methods, RA patients were also categorized into different groups based on different ESR and CRP levels: RA patients with increased ESR (>15 mm/h for men and >20 mm/h for women) and increased CRP (>8 mg/L), RA patients with increased ESR and normal CRP, RA patients with normal ESR and increased CRP, RA patients with normal ESR and normal CRP. As shown in Supplementary Figure 1A-D, results showed that there were no significant differences between miRNAs and these laboratory indicators in all subgroups. These results were also further confirmed by correlation analysis. The above results made us realise that circulating miR-22-3p and let-7a-5p may be independent predictive factors in RA.

DAS28 is one of the most widely used scoring system in clinical practice for the assessment of RA disease activity (28). Similarly, we did not find the correlation between DAS28 score and cir-

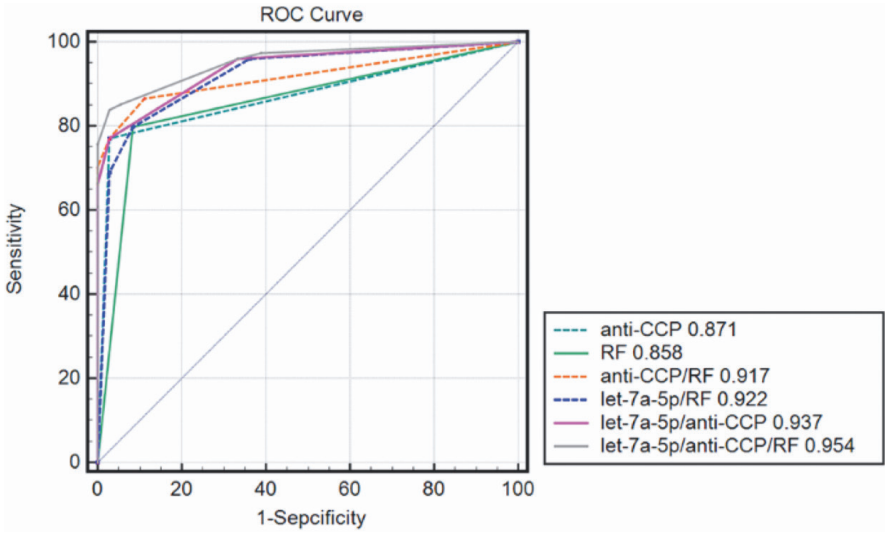

Fig. 3. Complementary diagnostic value of circulating let-7a-5p for RA. Complementary diagnostic value of circulating let-7a-5p combined with RF and/or anti-CCP between RA and HD was assessed using combined ROC analysis. Anti-CCP/RF represents the ROC curve of anti-CCP combined with RF; let-7a-5p/RF represents the ROC curve of let-7a-5p combined with RF; let-7a-5p/anti-CCP represents the ROC curve of let-7a-5p combined with anti-CCP; let-7a-5p/anti-CCP/RF represents the ROC curve of the combination of three indicators. Numbers represent the area under the curve.
Anti-CCP: anti-cyclic citrullinated peptide antibodies; RF: rheumatoid factor.

culating miR-22-3p or let-7a-5p (Suppl. Fig. S1E).

Circulating let-7a-5p has the complementary diagnostic value for RA

Since RF and anti-CCP are disease-specific antibodies and routinely used for RA diagnosis, also taking into account the independence of circulating miR-22-3p and let-7a-5p, we assessed the complementary diagnostic value of these miRNAs in RA patients to RF and/or anti-CCP. To rule out confounding factors, the univariable and multivariable logistic regression analysis were performed firstly. The results showed that combination of let-7a-5p, RF and anti-CCP may be the best combination that predicted the RA diagnosis (Suppl. Table S3). The pairwise comparison of AUCs also validated miR-22-3p was no helpful in improving the diagnostic capacity in RA (Suppl. Table S4).

Then, ROC curves were plotted (Fig. 3), and more details of ROC curves analysis were listed in Supplementary Table S5. By pairwise comparison of AUCs in various diagnostic models (Table III), we found that the diagnostic efficacy of the combination of let-7a-5p/anti-CCP/RF was higher than that of the combination of anti-CCP/RF (p=0.0263) and let-7a-5p/anti-CCP (p=0.0488). However, the difference between combined let-7a-5p/anti-CCP/RF group and combined let-7a-5p/RF group was not reach statistical significance (p=0.0806), although the combination of three indices had the largest AUC (AUC=0.954).

These results supported let-7a-5p in plasma had the complementary diagnostic value for RA.

Circulating miR-22-3p and let-7a-5p facilitate the diagnosis in RF- and/or anti-CCP-negative RA patients

In the clinic, RF- and/or anti-CCP-negative RA patients may easily lead to missed diagnosis or misdiagnosis. Hence, it is critical for seronegative (RF and anti-CCP double negative) RA patients to find the potential biomarkers to improve the rate of diagnosis. We defined RA patients as miRNA-positive or miRNA-negative by the optimum cut-off value described above (the optimum cut-off values of miR-22-3p and let-7a-5p in RA *vs*. HD subgroup are provided in Supplementary Table S2). Then the performances of circulating miR-22-3p and let-7a-5p in seronegative RA patients were determined. As presented in Table IV, The positive rates of circulating miR-22-3p in RF-negative and anti-CCP-negative RA patients were 73.3% (11/15) and 64.7% (11/17). More importantly, circulating miR-22-3p still demonstrated a 70% (7/10) prevalence in seronegative RA patients. Similarly, the positive rates of circulating let-7a-5p in RF-negative, anti-CCP-negative and both negative RA patients were 80% (12/15), 82.4% (14/17) and 80% (8/10), respectively. However, the performance of miR-22-3p combined with let-7a-5p was not found to be better than that of an individual miRNA.

Circulating miR-22-3p and let-7a-5p correlate with the clinical parameters of RA

Correlation analysis was performed to determine which clinical characteristics

Table III. Comparison of the diagnostic ability among RF (alone), anti-CCP (alone) and various combination indicators in RA *vs*. HD subgroup by evaluating the area under curve (AUC) using z-tests (numbers represent p-values).

Indicators	AUC	anti-CCP	RF	anti-CCP/RF	let-7a-5p/RF	let-7a-5p/anti-CCP	let-7a-5p/anti-CCP/RF
anti-CCP	0.871	-					
RF	0.858	0.6951	-				
anti-CCP/RF	0.917	0.0118*	0.0128*	-			
let-7a-5p/RF	0.922	0.1469	0.0012**	0.8893	-		
let-7a-5p/anti-CCP	0.937	0.0007***	0.0185*	0.3170	0.4906	-	
let-7a-5p/anti-CCP/RF	0.954	0.0003***	0.001**	0.0263*	0.0806	0.0488*	-

The *asterisk* indicates statistically significant results. *p<0.05, **p<0.01, ***p<0.001.

Table IV. The positive rate of miRNAs indicators in various subgroups of patients with rheumatoid arthritis (RA).

Subgroups	miR-22-3p	let-7a-5p	Combined miR-22-3p/let-7a-5p
Negative RF	73.3% (11/15)	80% (12/15)	73.3% (11/15)
Negative anti-CCP	64.7% (11/17)	82.4 (14/17)	58.8% (10/17)
Sero-negative	70% (7/10)	80% (8/10)	70% (7/10)

Numbers indicate the positive rate (the corresponding miRNA-positive persons/total examined). miRNA-positive depends on the corresponding miRNA optimal cut-off value described above.

Table V. Correlation between laboratory indicators and miRNAs in RA patients.

Characteristic	miR-22-3p		let-7a-5p	
	r	*p*	*r*	*p*
RF (IU/ml)	0.034	0.7719	-0.003	0.9807
anti-CCP (RU/ml)	0.011	0.9438	-0.051	0.7382
ESR (mm/h)	-0.030	0.8028	-0.019	0.8691
CRP (mg/l)	-0.094	0.4266	-0.050	0.6705
RBC (10^{12}/l)	0.041	0.7268	-0.027	0.8194
WBC (10^9/l)	-0.054	0.6457	-0.059	0.6097
NEUT (10^9/l)	-0.047	0.6856	-0.029	0.8033
MONO (10^9/l)	0.023	0.8428	-0.003	0.9777
LYMPH (10^9/l)	-0.037	0.7522	-0.084	0.4730
Hb (g/l)	0.251	0.0300*	0.170	0.1430
MCHC (g/l)	0.290	0.0115*	0.305	0.0078**
PLT (10^9/l)	-0.035	0.7642	-0.044	0.7058
MPV (fl)	-0.340	0.0030**	-0.316	0.0061**
MPC (pg)	-0.245	0.0353*	-0.272	0.0193*
Alb (g/l)	0.460	0.0013**	0.097	0.5145
Glo (g/l)	-0.026	0.8632	-0.065	0.6696
ALT (U/l)	-0.033	0.7810	-0.012	0.9213
AST (U/l)	0.045	0.7044	0.039	0.7421
γ-GT (U/l)	0.096	0.4434	0.077	0.5382
ALP (U/l)	-0.059	0.6363	-0.001	0.9947
CK (U/l)	0.417	0.0340*	-0.112	0.5845
TBIL (μmol/l)	0.384	0.0092**	-0.119	0.4243
DBIL (μmol/l)	0.336	0.0241*	-0.160	0.2813
IBIL (μmol/l)	0.401	0.0063**	0.034	0.8248
UREA (mmol/l)	-0.062	0.6431	-0.143	0.2810
CREA (μmol/l)	-0.075	0.5671	-0.055	0.6757
Glu (mmol/l)	0.063	0.7396	-0.379	0.0389*
TG (mmol/l)	0.006	0.9753	-0.111	0.5577
TCHO (mmol/l)	0.066	0.7290	-0.029	0.8775
HDL-C (mmol/l)	0.260	0.1652	0.209	0.2673
LDL-C (mmol/l)	-0.104	0.5844	-0.127	0.5022
VLDL-C (mmol/l)	-0.011	0.9523	-0.113	0.5514
Ca (mmol/l)	0.440	0.0245*	0.134	0.5155
Mg (mmol/l)	0.199	0.3296	0.066	0.7489
P (mmol/l)	0.244	0.2292	0.179	0.3807
AG (mmol/l)	0.405	0.0444*	-0.071	0.7286
CYSC (mg/l)	0.391	0.0183*	0.300	0.0710

RF: rheumatoid factors; anti-CCP: anti-cyclic citrullinated peptide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RBC: red blood cell count; WBC: white blood cell count; NEUT neutrophil count; MONO monocyte count; LYMPH: lymphocyte count; Hb: haemoglobin; MCHC: mean cell haemoglobin concentration; PLT: platelet count; MPV: mean platelet volume; MPC: mean platelet contents; Alb: albumin; Glo: globulin; ALT: Alanine aminotransferase; AST: Aspartate transaminase; γ-GT: glutamyl transpeptidase; ALP: alkaline phosphatase; CK: creatine kinase; TBIL: total bilirubin; DBIL: direct bilirubin; IBIL: indirect bilirubin; UREA: urea; CREA: creatinine; Glu: Glucose; TG: triglyceride; TCHO: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; Ca: Calcium; Mg: magnesium; P: Phosphorus; AG: anion gap; CYSC: cystatin C.
The *asterisk* indicates statistically significant results. *$p<0.05$, **$p<0.01$.

were related to circulating miR-22-3p and let-7a-5p (Table V). Data of laboratory indicators were collected and results showed that the levels of plasma miR-22-3p positively correlated with Hb, MCHC, Alb, CK, TBIL, DBIL, IBIL, Ca, AG and CYSC levels but negatively correlated with MPV and MPC, while the levels of plasma let-7a-5p positively correlated with MCHC but negatively correlated with MPV, MPC and Glu (Suppl. Fig. S2).

Considering that RA is a systemic inflammatory disease, serum samples were collected from RA patients to determine the serum cytokines, which including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-17, IFN-γ, IFN-α and TNF-α (Table VI). Correlation analysis between circulating miR-22-3p or let-7a-5p and cytokine levels was conducted and we found that there was a positive correlation between circulating miR-22-3p and serum IL-17 in RA patients.

Discussion

Circulating miRNAs can be quantified in almost all body fluids and especially in serum or plasma attributed to their stability and universality. As a non-invasive method, the detection of circulating miRNAs is considered to be a novel and valuable test method (24, 29-31). Based on these data, we anticipated that circulating miRNAs would be an ideal biomarker to differentiate RA patients from other populations. At the beginning of the study, miR-22-3p, miR-124-3p, miR-218-5p and let-7a-5p were included, as these miRNAs were likely to take part in RA pathogenesis based on our previous work (unpublished work) (19). MiR-218-5p and miR-124-3p were then excluded because no statistically difference was found between RA and HD or it was quite difficult to detect in plasma (data not shown). The results showed that miR-22-3p and let-7a-5p were significantly increased in the plasma of patients with RA in comparison with HD or patients with SLE and SS.

It has been demonstrated that miR-22-3p plays complicated roles in RA pathogenesis, and a potential clinical application has been shown. We and others have reported that decreased miR-22-3p

Table VI. Correlation between cytokines levels and miRNAs in RA patients.

Characteristics	miR-22-3p		let-7a-5p	
	r	*p*	*r*	*p*
IL-1β (pg/ml)	0.189	0.226	-0.0175	0.547
IL-2 (pg/ml)	0.049	0.7891	0.0069	0.970
IL-4 (pg/ml)	0.218	0.317	0.0801	0.716
IL-5 (pg/ml)	-0.089	0.617	-0.0433	0.808
IL-6 (pg/ml)	-0.108	0.5061	-0.0989	0.544
IL-8 (pg/ml)	-0.167	0.5513	-0.1399	0.619
IL-10 (pg/ml)	-0.064	0.7367	-0.141	0.457
IL-12 p70 (pg/ml)	-0.117	0.5778	-0.1095	0.602
IL-17 (pg/ml)	0.521	0.0006***	0.1276	0.517
IFN-γ (pg/ml)	0.221	0.8613	-0.615	0.961
IFN-α (pg/ml)	0.093	0.6259	-0.0022	0.998
TNF-α (pg/ml)	-0.116	0.6582	0.1864	0.474

IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.
The *asterisk* indicates statistically significant results. ***$p<0.001$.

in RA synovial tissue promoted FLS proliferation and proinflammatory cytokine production (19, 32). The levels of miR-22 in serum were increased in the susceptible individuals (pre-RA) (33) and low expression of miR-22 combined with increased miR-886 in serum had a high predictive value in the adalimumab treatment response (34). These observations all suggested that miR-22 may be a potential biomarker to predict RA development. Moreover, the increased circulating miR-22-3p in pre-RA also implied that miR-22-3p was associated with the early stages of disease development, which further support this possibility that circulating miR-22-3p could be a diagnostic biomarker of RA in clinical practice.

Adapted from the prior studies (35), circulating let-7a-5p was initially used as an internal reference to calculate the relative miRNA expression in our work. However, when we introduced cel-miR-39-3p as an external reference to the experiments, we unexpectedly found that there was a significant increase in the expression levels of let-7a-5p in plasma between RA and HD. Given that some studies showed that decreased let-7a facilitated IL-1β production in monocytes (21) and synovial fluid macrophage activation (20), we considered let-7a-5p participated in the pathogenesis of RA and thus likely led to the alteration of expression level in plasma. In fact, to our knowledge, this is the first report that altered expression of circulating let-7a-5p was observed in RA. Both miR-22-3p and let-7a-5p have been shown that the expression levels are decreased in the intracellular but increased in the extracellular spaces (19-21, 32). It is important to note that the extracellular miRNAs do not necessarily reflect the abundance of those within the cells. The extracellular and cellular miRNA profiles may differ due to the cellular selection mechanism for miRNAs release and expression from a multitude of cellular sources (36).

Several circulating miRNAs have been identified as potential diagnostic markers in various diseases, individually or as panels (37). Given that the early and accurate diagnosis of RA contributes to the improved outcome of RA (9), the diagnostic value of circulating miR-22-3p and let-7a-5p in RA were first assessed. The ROC curve analysis revealed that miR-22-3p and let-7a-5p in plasma had a good performance in the RA diagnosis. Circulating let-7a-5p showed the better discriminatory ability between RA and HD while miR-22-3p do better between RA and SLE, which indirectly supported let-7a-5p may be involved in the pathogenesis of SLE (38).

In addition to the diagnostic values of miRNAs themselves, the comparisons to the established biomarkers RF and anti-CCP were performed. Although none of the miRNAs achieves better specificity and sensitivity than RF or anti-CCP, circulating let-7a-5p still showed a good complementary diagnostic value to RF or anti-CCP (AUC=0.954). This discovery provided a possibility that miRNAs, individually or as panels, combined with RF and/or anti-CCP could improve the diagnostic capability of RA.

The other notable observation concerns the performance of circulating miRNAs in seronegative RA patients. Our results suggested that miR-22-3p shows a 70% positive rate and 80% for let-7a-5p in seronegative RA patients, revealing a high positive rate of miRNAs. All results above further supported miR-22-3p and let-7a-5p are of high clinical utility in RA patients, especially in seronegative RA individuals.

Finally, we assessed the relationship between circulating miR-22-3p or let-7a-5p and the clinical parameters of RA. Correlation analysis revealed that plasma miR-22-3p and let-7a-5p correlated well with several important biological markers. Previous reports have shown that changes in haemoglobin, serum bilirubin and mean platelet volume are strongly tied to the disease activity of RA (39-41). The correlation between circulating miRNAs and these indicators suggested circulating miR-22-3p and let-7a-5p may be associated with the clinical activity in patients with RA. Also, serum bilirubin and albumin are important indicators to indicate hepatic function. The links between circulating miR-22-3p and serum bilirubin or albumin indicated that miR-22-3p may play a role in the mechanism of hepatic injury in RA. Meanwhile, although some correlations were found between the investigated miRNAs and indicators including MPC, CK, Glu, Ca, AG and CYSC in RA, the observed relationships were not strong. Thus, their exact connection should be further investigated.

Regarding the inflammatory cytokines, we found circulating miR-22-3p showed strong positive correlation with IL-17, which predominantly secreted by T helper 17 cells (Th17). The imbalance of Th17 and Treg cells contributes to the RA onset (42). Although several investigators have suggested that miR-22 could drive Th17 responses in emphysema and inflammatory bowel disease (43, 44), there are not reported that the relationship between miR-22-3p and Th17 in RA, which show an interesting future direction of next research.

In conclusion, we find that circulating

miR-22-3p and let-7a-5p are significantly increased in RA patients compared to HD or patients with SLE and SS. Our findings support circulating miR-22-3p and let-7a-5p could be used for RA diagnosis, especially in seronegative RA patients. Moreover, correlation analysis reveals that circulating miR-22-3p and let-7a-5p may be related to the disease activity of RA and miR-22-3p may take part in the RA-associated hepatic injury or Th17 responses. Based on our research, we demonstrate that circulating miR-22-3p and let-7a-5p, individually or as panels, have a great potential application in clinical practice.

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