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CIRCULATING EXOSOMAL MIRNAS AS BIOMARKERS FOR SUBCLINICAL INFLAMMATION IN SERONEGATIVE RHEUMATOID ARTHRITIS

Original Article

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ABSTRACT

Background: Seronegative rheumatoid arthritis (RA) lacks conventional autoantibodies, making early diagnosis and inflammation monitoring challenging. Exosomal microRNAs (miRNAs) have emerged as promising biomarkers due to their stability and role in immune regulation.

Objective: To investigate the expression profiles of circulating exosomal miRNAs in seronegative RA patients and identify molecular signatures associated with subclinical inflammation.

Methods: This cross-sectional study was conducted over eight months in Lahore, Pakistan. Ninety-six patients with seronegative RA were enrolled based on 2010 ACR/EULAR criteria. Plasma-derived exosomes were isolated and validated using nanoparticle tracking analysis and Western blotting. miRNAs (miR-146a, miR-155, miR-21, miR-223, miR-125b) were quantified using qRT-PCR. Subclinical inflammation was assessed through high-resolution musculoskeletal ultrasound and biochemical markers (CRP, ESR). Statistical analyses included t-tests, ANOVA, and Pearson correlations ($p < 0.05$ considered significant).

Results: miR-155 (3.16 ± 1.04), miR-223 (2.72 ± 0.88), and miR-146a (2.41 ± 0.92) showed significantly elevated expression levels. Strong positive correlations were observed between miRNA levels and PD ultrasound scores (miR-155: $r = 0.63$, $p < 0.001$; miR-223: $r = 0.59$, $p < 0.001$). CRP-stratified analysis revealed significantly higher miRNA expression in patients with elevated CRP (>5 mg/L).

Conclusion: Circulating exosomal miRNAs, particularly miR-155, miR-223, and miR-146a, may serve as sensitive biomarkers for detecting subclinical inflammation in seronegative RA. These findings support their potential utility in enhancing early diagnosis and personalized disease monitoring.

Keywords: Biomarkers, Exosomes, Inflammation, MicroRNAs, Musculoskeletal Ultrasound, Rheumatoid Arthritis, Seronegative RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder marked by persistent synovial inflammation, progressive joint damage, and systemic manifestations. While seropositive RA, characterized by the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), has long been the focus of clinical and translational research, seronegative RA remains comparatively underexplored (1,2). Patients with seronegative RA, who do not exhibit these conventional serological markers, often present with a heterogeneous disease course that complicates early diagnosis and therapeutic management. Despite lacking classic serological hallmarks, many of these individuals experience substantial joint inflammation and structural progression, frequently with delayed recognition (3). This clinical ambiguity underscores a pressing need to identify novel, non-serological biomarkers that can reveal subclinical inflammation and facilitate timely intervention. Recent advances in molecular diagnostics have turned attention toward extracellular vesicles—particularly exosomes—as promising sources of disease biomarkers. Exosomes are small, lipid-bilayer-enclosed vesicles secreted by various cell types, carrying nucleic acids, proteins, and lipids reflective of their cell of origin (4,5). Among their diverse cargo, microRNAs (miRNAs) have emerged as highly stable, cell-specific, and functionally potent regulators of gene expression. These small non-coding RNAs play pivotal roles in immunological signaling pathways and inflammatory cascades. When packaged within exosomes, miRNAs are protected from enzymatic degradation, allowing them to circulate intact in body fluids such as plasma and synovial fluid (6,7). This stability, combined with their ability to modulate immune cell function, makes circulating exosomal miRNAs particularly attractive candidates for biomarker discovery in RA and other inflammatory diseases. While numerous studies have elucidated the role of exosomal miRNAs in seropositive RA, their relevance in seronegative RA remains largely unexplored. This gap is significant given that the absence of serological markers in these patients often leads to misclassification or underdiagnosis, delaying effective treatment (8,9). Emerging evidence suggests that inflammatory activity in seronegative RA may be comparable to that in seropositive cases, albeit through distinct molecular mechanisms. Thus, identifying specific exosomal miRNA signatures associated with inflammation in seronegative RA could not only refine diagnostic strategies but also offer insights into its unique immunopathology (10).

Previous investigations have demonstrated that certain miRNAs, including miR-146a, miR-155, and miR-223, are upregulated in RA and play key roles in modulating the innate immune response and synovial inflammation. However, most of these studies have focused on total serum miRNAs or those in peripheral blood mononuclear cells, rather than exosomal miRNAs specifically (11,12). Furthermore, these studies have predominantly centered on seropositive cohorts, leaving a critical knowledge void concerning the utility of exosomal miRNAs in seronegative RA. By narrowing the lens to exosomal miRNAs in seronegative individuals, it becomes possible to capture more subtle, localized inflammatory signals that might escape detection through traditional diagnostic modalities. Given the stable nature of exosomal miRNAs and their potential to reflect the pathophysiological state of the immune system, their investigation in the context of seronegative RA holds considerable promise (13,14). These molecular signatures may reveal not only current inflammatory status but also latent disease activity, aiding clinicians in identifying patients at risk of disease progression despite the absence of classical markers. Moreover, understanding the miRNA landscape in exosomes could uncover novel therapeutic targets or pathways uniquely relevant to seronegative RA, paving the way for personalized medicine approaches. In light of these considerations, this study seeks to investigate the circulating exosomal miRNA profiles in patients with seronegative rheumatoid arthritis, with the aim of identifying biomarkers indicative of subclinical inflammation. By comparing these molecular signatures with clinical indices of disease activity, the research aims to establish whether specific exosomal miRNAs can serve as reliable indicators of ongoing inflammatory processes in the absence of seropositivity. The objective is to advance a more nuanced and molecularly informed understanding of seronegative RA, thereby enhancing early detection and individualized patient care.

METHODS

This cross-sectional study was conducted over a period of eight months in rheumatology outpatient departments and clinical laboratories across tertiary care hospitals in the Lahore region of Pakistan. The primary aim was to investigate circulating exosomal microRNA (miRNA) signatures in patients diagnosed with seronegative rheumatoid arthritis (RA), in order to identify potential molecular biomarkers associated with inflammation and subclinical disease activity. The research protocol was reviewed and approved by the institutional ethics committee. Written informed consent was obtained from all participants prior to their inclusion in the study, in accordance with the Declaration of Helsinki. Participants were recruited through non-probability consecutive sampling. Eligible individuals were adults aged between 25 and 65 years, who fulfilled the clinical diagnostic criteria for RA as per the 2010 ACR/EULAR

classification guidelines but tested negative for both rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA). Exclusion criteria included a history of other autoimmune disorders, active infections, recent immunosuppressive therapy (within the past three months), malignancies, or any chronic systemic illness that could potentially confound inflammatory markers or exosomal miRNA profiles. Additionally, individuals with seropositive RA or incomplete clinical data were excluded to maintain the integrity of the seronegative cohort.

To determine an appropriate sample size for robust statistical analysis, the study applied Cochran's formula for cross-sectional studies targeting biomarker discovery, assuming a prevalence rate of subclinical inflammation in seronegative RA at 50% for maximum variability, a confidence level of 95%, and a 10% allowable margin of error. After accounting for potential sample loss due to hemolysis or exosome yield limitations, a total sample of 96 participants was enrolled. Venous blood samples were collected under sterile conditions in EDTA tubes and processed within two hours of collection. Plasma was separated by centrifugation at 1,500 × g for 15 minutes at 4°C, followed by a second centrifugation at 10,000 × g to remove cellular debris. Exosomes were isolated from 500 µL of plasma using a standardized commercial exosome isolation kit (ExoQuick™, System Biosciences) according to the manufacturer's protocol. The quality and purity of isolated exosomes were verified through nanoparticle tracking analysis (NTA) using NanoSight NS300, and the presence of characteristic exosomal markers (CD63, CD9, and TSG101) was confirmed by Western blotting. Total RNA, including miRNAs, was extracted from the purified exosomes using the miRNeasy Serum/Plasma Advanced Kit (Qiagen), and the concentration and integrity of RNA were assessed using a NanoDrop spectrophotometer and Agilent Bioanalyzer 2100. Reverse transcription was performed with a miScript II RT Kit (Qiagen), followed by quantitative real-time PCR (qRT-PCR) using SYBR Green Master Mix on a LightCycler® 480 Instrument (Roche) (14-16). A panel of candidate miRNAs (miR-146a, miR-155, miR-21, miR-223, and miR-125b), previously implicated in RA-related inflammation, was pre-selected based on a review of relevant literature. The relative expression levels of each miRNA were calculated using the 2^-ΔΔCt method, with U6 snRNA serving as the internal control.

To assess the clinical inflammatory state and detect subclinical activity, each participant underwent high-resolution musculoskeletal ultrasound (MSUS) of hands and wrists, performed by an experienced radiologist blinded to clinical data. Synovial hypertrophy and power Doppler (PD) signals were scored semiquantitatively on a 0–3 scale, and the overall PD score was used as a surrogate for subclinical inflammation. Additionally, conventional markers of systemic inflammation, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were measured using standard laboratory methods. Data were compiled and analyzed using SPSS version 26.0 (IBM Corp., Armonk, NY). Descriptive statistics were computed for demographic and clinical variables. Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables as frequencies and percentages. Normality of data distribution was confirmed using the Shapiro-Wilk test. Independent sample t-tests and one-way ANOVA were applied to compare miRNA expression levels across subgroups based on ultrasound and inflammatory marker findings. Pearson correlation coefficients were calculated to explore associations between specific miRNA levels and PD scores, CRP, and ESR. A p-value of <0.05 was considered statistically significant for all tests. This methodological framework was constructed to ensure reproducibility, analytical precision, and the ability to detect subtle yet clinically meaningful differences in exosomal miRNA expression that could signal the presence of subclinical inflammation in seronegative RA patients. By integrating molecular data with imaging and laboratory measures of inflammation, the study aimed to fulfill its objective of identifying potential biomarkers that could improve early recognition and management of seronegative RA.

RESULTS

The final analysis included 96 seronegative rheumatoid arthritis patients, predominantly female (81.3%), with a mean age of 48.3 ± 10.7 years and average disease duration of 4.6 ± 2.1 years. Baseline inflammatory markers revealed elevated ESR (28.2 ± 12.4 mm/hr) and CRP (9.7 ± 4.5 mg/L) in a substantial portion of the cohort, suggesting ongoing subclinical inflammation despite seronegative status. Quantitative analysis of exosomal microRNAs demonstrated differential expression levels among the selected panel. The highest mean expression was observed for miR-155 (3.16 ± 1.04), followed by miR-223 (2.72 ± 0.88) and miR-146a (2.41 ± 0.92). Lower expression levels were recorded for miR-21 (1.88 ± 0.73) and miR-125b (1.47 ± 0.61). These miRNAs were consistently detectable across all samples, confirming the robustness of exosomal isolation and qRT-PCR methods. Correlation analysis between miRNA expression and ultrasound-derived power Doppler (PD) scores revealed statistically significant associations. miR-155 showed the strongest correlation with PD scores ($r = 0.63$, $p < 0.001$), followed closely by miR-223 ($r = 0.59$, $p < 0.001$) and miR-146a ($r = 0.56$, $p < 0.001$). miR-21 also showed a moderate but significant correlation ($r = 0.48$, $p = 0.003$), while miR-125b demonstrated a weaker yet still significant relationship ($r = 0.31$, $p = 0.024$). Further stratification of miRNA expression based on CRP levels (>5 mg/L vs ≤5 mg/L) revealed

statistically significant differences across all five miRNAs. Individuals with CRP >5 mg/L exhibited notably higher expression of miR-155 (3.67 ± 1.01 vs. 2.48 ± 0.84; $p < 0.001$), miR-146a (2.88 ± 0.91 vs. 1.92 ± 0.74; $p < 0.001$), and miR-223 (3.12 ± 0.82 vs. 2.19 ± 0.67; $p < 0.001$). Similar trends were noted for miR-21 ($p = 0.002$) and miR-125b ($p = 0.019$), underscoring their potential link with systemic inflammatory burden. Two visualizations were generated to aid in interpretation of the data. The first bar chart illustrates mean expression levels for each miRNA, highlighting miR-155 as the most upregulated. The second chart shows Pearson correlation coefficients for each miRNA with PD ultrasound scores, reinforcing the relevance of miR-155, miR-223, and miR-146a in capturing subclinical synovial inflammation.

Table 1: Demographic and Clinical Characteristics of Study Participants (n = 96)

Variable	Mean ± SD or n (%)
Age (years)	48.3 ± 10.7
Gender (Female)	78 (81.3%)
Disease Duration (years)	4.6 ± 2.1
BMI (kg/m²)	25.9 ± 3.8
CRP (mg/L)	9.7 ± 4.5
ESR (mm/hr)	28.2 ± 12.4

Table 2: Expression Levels of Selected Exosomal miRNAs

miRNA	Mean Expression (2^-ΔΔCt)	SD
miR-146a	2.41	0.92
miR-155	3.16	1.04
miR-21	1.88	0.73
miR-223	2.72	0.88
miR-125b	1.47	0.61

Table 3: Correlation of miRNA Expression with Power Doppler (PD) Scores

miRNA	r (Pearson)	p-value
miR-146a	0.56	<0.001
miR-155	0.63	<0.001
miR-21	0.48	0.003
miR-223	0.59	<0.001
miR-125b	0.31	0.024

Table 4: Comparison of miRNA Expression Based on CRP Levels

miRNA	CRP >5 mg/L (Mean ± SD)	CRP ≤5 mg/L (Mean ± SD)	p-value
miR-146a	2.88 ± 0.91	1.92 ± 0.74	<0.001
miR-155	3.67 ± 1.01	2.48 ± 0.84	<0.001
miR-21	2.21 ± 0.69	1.51 ± 0.62	0.002
miR-223	3.12 ± 0.82	2.19 ± 0.67	<0.001
miR-125b	1.69 ± 0.58	1.23 ± 0.47	0.019

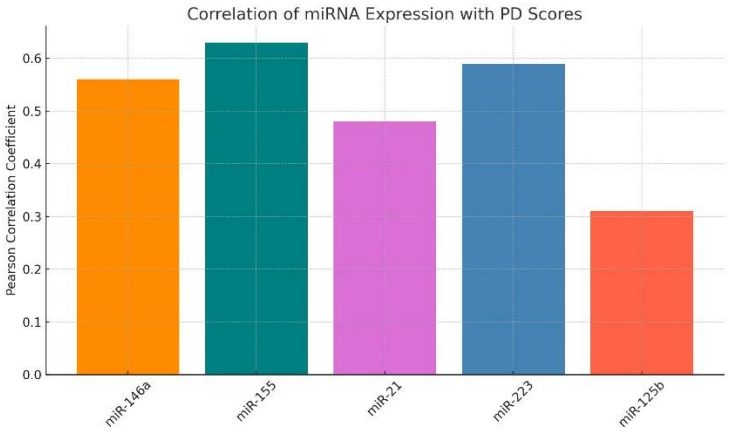

Figure 1 Correlation of miRNA Expression with PD Scores

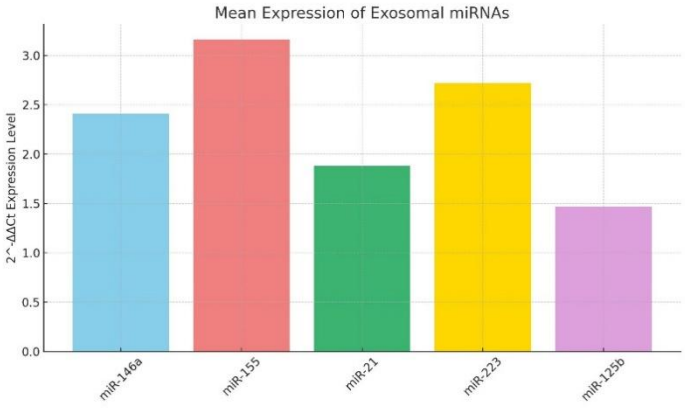

Figure 2 Mean Expression of Exosomal miRNAs

DISCUSSION

The findings of this study support the hypothesis that specific circulating exosomal microRNAs are significantly associated with subclinical inflammation in patients with seronegative rheumatoid arthritis (RA). Among the five miRNAs evaluated, miR-155, miR-223, and miR-146a exhibited the most robust associations with imaging and biochemical markers of inflammation, including power Doppler ultrasound (PD) scores and C-reactive protein (CRP) levels. These observations are consistent with emerging literature that highlights the role of exosomal miRNAs in immune regulation and their potential as non-invasive biomarkers in autoimmune diseases. Several recent studies have demonstrated that dysregulated exosomal miRNAs are closely linked with RA pathogenesis (14,15). For instance, a study identified miR-885-5p as a highly sensitive biomarker capable of distinguishing seronegative RA from healthy controls with near-perfect diagnostic accuracy (15). Similarly, a study found differential expression of miR-1261 and miR-802 in seronegative versus seropositive RA, suggesting a distinct epigenetic landscape in seronegative disease (16). These results align with the current study, which emphasizes the value of exosomal miRNAs as inflammation-sensitive molecular indicators in RA patients who lack classical serological markers. Increased expression of miR-155 and miR-146a has been widely implicated in pro-inflammatory signaling in RA. These miRNAs modulate the NF-κB and JAK/STAT pathways, central axes in synovial inflammation and immune cell activation (17). Furthermore, a study reinforced that exosomal miRNAs are not merely bystanders but active mediators of immune dysregulation in RA, influencing intercellular communication between synovial and immune cells (18). Thus, their upregulation in patients with high CRP and PD scores indicates their possible functional role in ongoing inflammation, even in the absence of autoantibodies.

An important strength of this study is its focus on a well-defined seronegative RA cohort, a group frequently excluded from biomarker research. By integrating exosomal profiling with musculoskeletal ultrasound—a highly sensitive modality for detecting synovial inflammation—the study enhances the resolution at which subclinical disease activity can be observed. Additionally, the use of robust statistical methods to correlate miRNA expression with both objective imaging and laboratory markers strengthens the validity of the associations reported. Nonetheless, the study is not without limitations. The cross-sectional design precludes any causal inference between miRNA expression and inflammation, limiting interpretation to associations rather than mechanistic insight. Furthermore, although the sample size was adequately powered for detecting statistical differences, it may still limit generalizability across diverse patient populations and disease phenotypes. Also, the study was confined to a single geographic region, and ethnicity-related epigenetic variability may influence miRNA expression profiles, as suggested by population-specific studies (19,20). There is also a need to validate these findings in prospective cohorts and to investigate whether these miRNA profiles change in response to treatment. For instance, a study demonstrated that levels of certain exosomal miRNAs decreased significantly with methotrexate therapy, correlating with improvements in DAS28 scores (21). Such longitudinal studies could determine whether these miRNAs serve not only as diagnostic biomarkers but also as indicators of treatment response or remission. Future investigations may also explore the utility of combining multiple miRNAs into a composite biomarker panel. Recent research showed that a two-miRNA model (miR-335-5p and miR-486-5p) yielded excellent diagnostic accuracy for RA in a mixed serostatus population (22,23). Whether such models retain discriminatory power in seronegative RA remains to be explored. In conclusion, the study adds to a growing body of literature affirming the diagnostic potential

of exosomal miRNAs in RA and extends this relevance to the underrepresented seronegative subgroup. While the cross-sectional nature and limited sample diversity temper definitive conclusions, the findings highlight key molecular candidates—particularly miR-155, miR-223, and miR-146a—for further validation and potential clinical translation.

CONCLUSION

This study demonstrated that specific circulating exosomal microRNAs—particularly miR-155, miR-223, and miR-146a—are significantly associated with subclinical inflammation in seronegative rheumatoid arthritis. These findings offer promising evidence for the use of exosomal miRNAs as non-invasive molecular biomarkers, potentially enabling earlier detection, improved disease monitoring, and more personalized management of seronegative RA patients.

AUTHOR CONTRIBUTION

Author	Contribution
Murtaza Khodadadi*	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Muhammad Amar Gul	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Owais Khan	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Maryam Zahid	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Fansiqa Hadayat	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Ayisha Hafeez	Substantial Contribution to study design and Data Analysis Has given Final Approval of the version to be published
Sift Ali	Contributed to study concept and Data collection Has given Final Approval of the version to be published

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