Research article

Evaluating microRNA-499 expression and serum IL-17, ACCP and PADI4 cytokine levels in rheumatoid arthritis patients undergoing anti-TNF alpha therapy

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ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by joint inflammation and damage. While anti-TNF-alpha therapies like infliximab have revolutionized RA, treatment response varies among patients. Identifying reliable biomarkers for treatment response is crucial for optimizing therapy and patient outcomes. MicroRNA-499 has been implicated in autoimmune diseases, and IL-17 is a key mediator in RA pathogenesis. Additionally, PADI4 and ACCP are linked to RA autoimmunity. This study aimed to investigate the potential of microRNA-499 expression, serum levels of IL-17, PADI4 and ACCP as biomarkers for predicting and monitoring treatment response in RA patients undergoing anti-TNF-α (infliximab) therapy.

Methodology: In this case-control research, blood samples from 100 Iraqi patients with RA and 100 controls were collected. RNA was extracted and the levels of miRNA499 were quantified using real-time PCR. The serum IL-17, PADI4 and ACCP levels were determined using ELISA. Patients were sub-grouped as responders and non-responders based on their responses to anti-TNF-α (infliximab) treatment.

Results: The mean age of the patients in this study was 46.22±11.33 years. The occurrence of RA was higher in age group (31-45) years. There was a significant association between RA, smoking and BMI. miRNA-499 expression was significantly higher in RA patients versus controls (1.1 folds in patients compared to 0.52 in control group). The serum levels of IL-17, PADI4 and ACCP was significantly higher in RA patients than controls (54.3ng/L, 2.52 ng/ml and 2.15 U/ml) vs (40.35ng/ L, 1.78 ng/ml and 1.06 U/ml) respectively. Subdividing of patients showed that miRNA-499 expression and serum IL-17, PADI4 and ACCP to be significantly higher in non-responders patients than responders.

Conclusion: MicroRNA-499 expression, serum levels of IL-17, PADI4 and ACCP were found to be significantly higher in RA patients compared to control group as well as in non-responding than responding patients.

Keywords: MicroRNA-499; IL-17; PADI4; ACCP; Rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease of unknown etiology. Emerging evidence highlights the significance of epigenetic modifications in regulating RA pathogenesis (1-4). Major epigenetic mechanisms involve DNA methylation, histone protein modifications, and alterations in gene expression induced by microRNAs and other non-coding RNAs (5). MicroRNAs (miRNAs) are small non-coding RNA molecules (~22 nucleotides long) that bind to the 3′ untranslated region (UTR) of their respective target mRNAs bringing about inhibition of gene expression. Contrasting, a recent report shows that under certain conditions, miRNAs can activate and regulate gene expression genes due to their unique shuttling mechanism (6).

Several human disorders have been linked to dysregulation of miRNA post-transcriptional inhibition (7). Among the inflammatory cytokines suppressed by miRNA-499 are IL-17 receptor βIL-17R, IL-2R, IL-18R, IL-23, IL-6, IL-2, and IL-21, as well as PADI4. These cytokines have been shown to play an important role in the onset of rheumatoid arthritis (8).

Myeloid, mast, and T-helper-17 cells secrete the pro-inflammatory cytokine interleukin-17 (IL-17). IL-17 has the ability to stimulate various cell types, including chondrocytes, osteocytes, synovial fibroblasts, and macrophages, to release and produce more pro-inflammatory cytokines. The inflammatory response in rheumatoid arthritis is merely intensified by this cascade of cytokine production (9). In addition to RA, IL-17 is also involved in the development and progression of a number of other chronic inflammatory and autoimmune diseases. Recent studies have shown that precise and efficient regulation of IL-17 signaling has the potential to effectively manage the vast majority of these disorders (10).

Peptidyl arginine deiminase type 4 (PADI4) enzyme is responsible for the catalysis of amino acid arginine into citrulline during post-translational deamination (11). Autoantibodies directed against citrullinated

proteins (Anti-cyclic citrullinated peptides ACCP) are highly specific for RA and suggest the involvement of PADIs in the pathogenesis of RA (12). This study aimed to assay the serum levels of the inflammatory cytokines IL-17, PADI4 and ACCP in RA patients and correlate it to microRNA-499 responsiveness to TNF alpha inhibitor treatment, which may be useful to optimizing treatment and reduce adverse side-effects.

MATERIALS AND METHODS

Study design

This case-control study undertaken from January 2022 to September 2022, involved 100 patients diagnosed with rheumatoid arthritis and who were being treated with TNF alpha inhibitors (infliximab) for at least 6 months. The patients were confirmed as having rheumatoid arthritis by Rheumatologists at the Rheumatology units of Baghdad Teaching Hospital and Al-Yarmouk Teaching Hospital, Baghdad, Iraq. Details like age, weight, and length of illness, smoking and patient medication dosages were gathered. A control group consisting of 100 individuals who appeared to be healthy was also included in the study.

The EULAR/ACR criteria, 2010 (13), was used to further group the patients based on their responses to treatment. The CDAI was calculated using the Rhumahlper app, and it was then used to assess clinical disease activity, including remission. Based on their CDAI, the patients were categorized into clinical subgroups (14). Over a maximum of six months, the treatment response was evaluated and classified using the EULAR response criteria (Table 1).

Table 1: Treatment response according to EULAR criteria

EULAR response criteria	Interpretation
CDAI < 10	Responders
CDAI >10	Non-responders

The study obtained ethical approval and informed permission from each subject in line with the guidelines outlined in the declaration of Helsinki. The Institutional Review Board (IRB) provided the necessary ethical agreement for the study in Al-Nahrain University-College of Medicine (No. 20211045 dated 30/12/2021).

Sample collection

Three milliliters of venous blood drawn from each participant was transferred to gel tubes for serum separation. For RNA extraction 0.4 mL of serum was added to 0.6 mL of TRIzol™ reagent and the remaining serum (stored at -20 C^{o}) was used in assaying the serum levels of IL-17, PADI4 and ACCP.

Quantitative Real-time PCR to detect miRNA-499

The RNA from the samples was isolated using the TRIzol™ reagent protocol. Synthesis of cDNA was performed using GoScript ™ reverse transcription system kit from Promega (USA) and designed microRNA specific stem-loop primers. Subsequently, the concentration of extracted cDNA was measured using a Quantus Fluorometer (Promega, USA) ensuring the quality of the samples for further analysis and downstream applications. miRNA-499 expression was measured using quantitative real-time polymerase chain reaction (PCR). The RT-qPCR primers were designed using Primer 3 software based on miRNA cDNA sequences obtained from the NCBI GenBank database. The primer sequences designed are presented in Table 2.

Table 2: Primers sequences used in this study

Primer Name	Sequence 5`-3`	Annealing Temp.
miR-499a-5p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTC GCACCAGAGCCAACAAACAT	55 °C
miR-499a-5p-F1	GGGGTTAAGACTTGCAGTG	
RNU43_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTC GCACCAGAGCCAACAATCAG	
RNU43_F	GTGAACTTATTGACGGGCG	
Universal Reverse primer	GTGCAGGGTCCGAGGT	

Table 3: The thermal profile of RT-PCR for miRNA expression

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:15	40
Annealing	55	00:15	
Extension	72	00:15	

The expression of miRNA-499a was determined by qPCR using the BRYT Green GoTaq qPCR master mix (Promega, USA) with the RNU43 gene was used as a house-keeping gene. The cycling conditions used are as given in Table 3. miRNA fold expression was calculated using the formula:

Folding $=2^{-\Delta\Delta CT}$

ΔCT = CT gene - CT Housekeeping gene

$\Delta\Delta CT$ = ΔCT Treated or Control – Average ΔCT Control

Serum levels of IL-17, PADI4 and ACCP

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the IL-17, PADI4 and ACCP levels in serum. Serum IL-17 levels were measured using a kit from Bioassay Technology Laboratory, China and the ELISA kit procured from Sun Long Biotech, China was utilized to measure human PADI4 and ACCP levels. The tests were carried out according to the manufacturer's instructions.

Statistical analysis

The statistical analyses were conducted using SPSS software version 25.0 from SPSS, Chicago. Categorical variables were presented as numbers and percentages, and their analysis was conducted using the Chi-square test. The diagnostic value of miR-499a, IL-17, PADI4 and ACCP in discriminating between the patient and control groups was assessed using ROC curves. Spearman's correlation test examined probable correlations of the measured markers with other continuous variables as well as between each other. A p-value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Demographic characteristic of study population

The average age of the rheumatoid arthritis patients in the study was 46.22±11.33 years, which was comparable to the control group's age of 45.1±11.95 years and statistically showing no significant difference (Table 4)**.** When the study population was divided into age groups, it was seen that patients in the age group of 31-45 years were significantly more common, followed by patients aged between 46-60 years (Table 4). It was also observed that RA patients had significantly higher BMI than the control group (27.6±4.77 kg/m2 vs. 25.31±4.0 kg/m2). The frequency of smokers among patients and control group was 35% and 19%, respectively, with a significant difference. Similarly, 32% of patients were found to have a familial history of RA (Table 4).

Table 4: Demographic characteristics of the study population

Variables	**Patients (n=100)**	**Controls (n=100)**	***p*-value**
Age, years			
Mean ±SD	46.22±11.33	45.1±11.95	0.059
Range	20-75	18-75	
16-30	9 (9%)	15 (15%)	
31-45	42 (42%)	31(31%)	**0.039**
46-60	35 (35%)	48 (48%)	
61-75	14 (14%)	6(6%)	
Age, years			
Mean ±SD			
Female	46.19±11.52-	41.91±11.52-	0.296
Male	44.16±10.69	46.06±11.88	0.095
Sex			
Male	25(25%)	31(31%)	0.345
Female	75(75%)	69(69%)	
Body Mass Index (BMI), kg/m^2			
Mean ±SD	27.6±4.77	25.31±4.0	**<0.001**
Range	17.3-45.55	16.37-37.0	
Underweight	6 (6%)	4(4%)	
Normal weight	24(24%)	46 (46%)	**0.005**
Overweight	40 (40%)	35 (35%)	
Obese	30 (30%)	15 (15%)	
Smoking			
No	65(65%)	81(81%)	**0.011**
Yes	35(35%)	19(19%)	
Family history			
No	68(68%)	100 (100%)	**<0.001**
Yes	32(32%)	0 (0%)	

Expression of miRNA-499a

The results showed a higher significant expression of miRNA-499a in serum of RA patients than those in healthy individuals. The relative median expression of miRNA-499a in RA patients was 1.1 folds (range= 00-211.69 folds), while it was 0.52 folds (range= 0.01 to 43 folds) in the control group (Fig. 1).

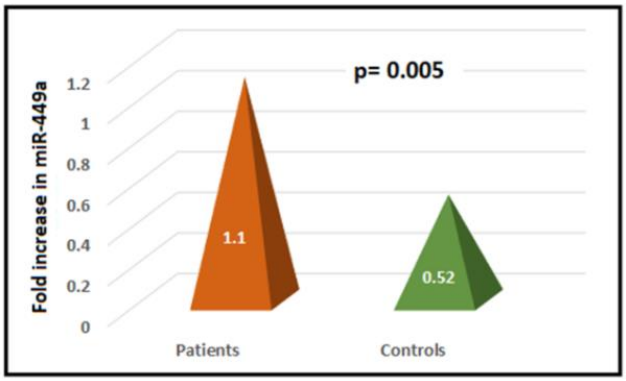

Fig. 1: Median folds of miRNA-499a in patients and control group

Diagnostic value of miRNA-499a

The ROC curve analysis revealed that the area under the curve (AUC) value to be 0.616, 95% CI=0.537-0.695, p=0.005. The sensitivity and specificity of the test at the cut off value of miRNA-499a= 0.66 folds were 62% and 67% respectively (Fig. 2).

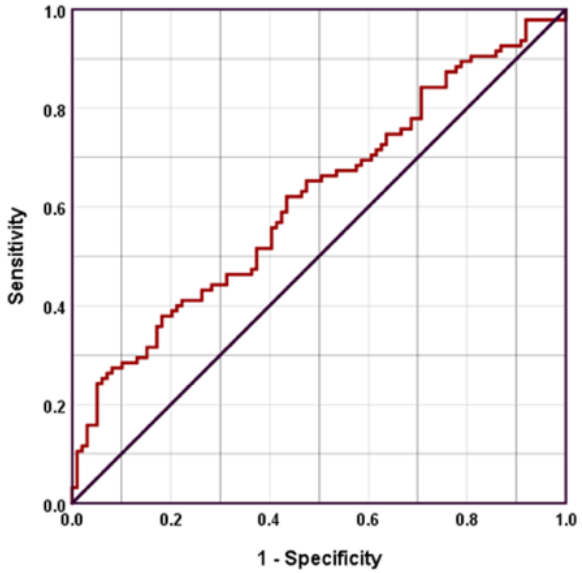

Fig. 2: Receiver operating curve analysis for miRNA-499a in patients and control group

Serum levels of IL-17, PADI4 and ACCP in studied groups

The median serum level of IL-17 in cases (54.3ng/L) was significantly higher than in the control group (40.35ng/ L) ($p < 0.001$). Moreover, the median serum level of PADI4 and ACCP in patients were (2.52ng/ml and 2.15U/ml) respectively which were higher than those of control group (1.78ng/ml and 1.46U/ml respectively) with a significant difference (Table 5).

Diagnostic values of IL-17, PADI4 and ACCP

The diagnostic value of IL-17 in distinguishing between patients with RA and the control group was evaluated using a Receiver Operating Characteristic (ROC) curve. The test exhibited a sensitivity of 80% and a specificity of 71%. For PADI-4, the AUC was 0.852, 95%CI= 0.793-0.911, p <0.001. The sensitivity and specificity of the test at the cut off value of PADI-4= 2.12 ng/ml was 83% and 80%, respectively. For ACCP, the AUC was 0.655, 95% CI= 0.570-0.740, p <0.001. The sensitivity and specificity of the test at cut off value of ACCP= 7.81 U/ml was 80% and 77%, respectively (Table 6)

Association of clinical factors with early clinical responsiveness

The average number of infliximab doses and length of treatment for responders and non-responders, respectively, were 4.76±1.23 doses and 4.64±1.69 months and 4.48±1.54 doses and 4.68±1.21 months, respectively. Initial CDAI scores varied from 4.0–13 for responsive respondents (with a mean ± standard deviation of 8.12±2.13), and from 10–30 for non-responders (mean ± standard deviation of 20.22±5.75), indicating a statistically significant difference (Table 7).

miR-499a expression in responder and non-responder patients

The relative median expression of this miRNA-499a was 0.82 folds (range= 0.3-82.5 folds) in responder patients compared to 1.27 folds (range= 0.0 to 211.69 folds) in non-responder patients (Fig. 3).

Table 5: Serum level of IL-17, PADI4 and ACCP in RA patients and control group

Variables	Patients (n=100)	Control group (n=100)	*p*-value
IL-17, ng/L			
Mean ±SD	68.16±40.96	44.08±19.23	**<0.001**
Median	54.3	40.35	
Range	36.35-207.48	21.28-120.2	
PADI-4, ng/ml			
Mean ±SD	2.86±1.36	1.96±0.77	**<0.001**
Median	2.52	1.78	
Range	1.5-9.38	0.76-5.63	
ACCP, U/ml			
Mean ±SD	16.04±20.33	1.6±0.74	**<0.001**
Median	2.15	1.06	
Range	0.14-67.84	0.81-5.39	

Table 6: Diagnostic value for IL-17, PADI4 and ACCP in patients with RA and control group

Marker	AUC, 95%CI	Sensitivity	Specificity	Cut off value	p-value
PADI-4	0.852, 0.793-0.911	83%	80%	2.12 ng/ml	<0.001
IL-17	0.793, 0.726-0.859	80%	71%	46.91 ng/L	<0.001
ACCP	0.655, 0.570-0.740	60%	57%	1.54 U/ml	<0.001

Table 7: Association of clinical factors with early clinical responsiveness

Variables	Responsive (n=50)	Non-responsive (n=50)	*p*-value
Number of doses of infliximab			
Mean ±SD	4.76±1.23	4.48±1.54	0.746
Range	2.0-7.0	2.0-6.0	
Duration of treatment, months			
Mean ±SD	4.64±1.69	4.68±1.21	0.622
Range	3.0-6.0	2.0-6.0	
Initial CDIA score			
Mean ±SD	8.12±2.13	20.22±5.75	**<0.001**
Range	4.0-13	10-30	

SD: standard deviation

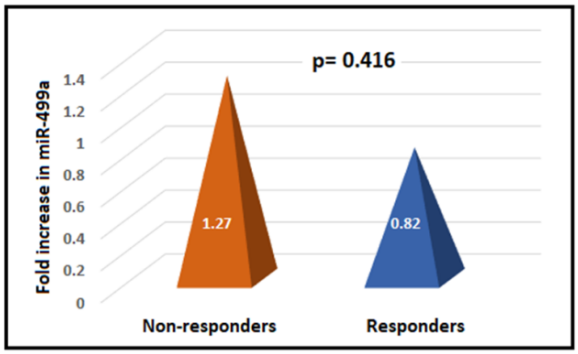

Fig. 3: Median folds of miRNA-499a in responder and non-responder patients

Table 8: Serum levels of IL-17, PADI4 and ACCP in patients

Variables	Responder (n=50)	Non- responder (n=50)	*p*-value
IL-17, ng/L			
Mean ±SD	62.21±36.56	75.60±45.25	**0.006**
Median	50.83	59.16	
Range	36.35-207.84	37.69-205.39	
PADI-4, ng/ml			
Mean ±SD	2.59±1.05	3.21±1.61	**0.001**
Median	2.4	2.69	
Range	1.50-8.61	1.89-9.38	
ACCP, U/ml			
Mean ±SD	10.34±12.61	20.61±24.02	0.570
Median	1.92	4.17	
Range	0.35-31.62	0.14-67.84	

Table 9: Correlation of miRNA-499, IL-17, PADI4 and ACCP with other variables

Variables	PADI-4		IL-17		ACCP		MiR-499a	
	r	p-value	r	p-value	r	p-value	r	p-value
Age	0.087	0.244	0.117	0.118	**0.178**	**0.018**	0.045	0.532
Weight	0.083	0.269	0.005	0.951	0.138	0.065	**0.146**	**0.042**
Height	0.102	0.174	0.046	0.543	0.022	0.768	0.012	0.871
BMI	0.040	0.590	-0.015	0.841	0.119	0.111	**0.159**	**0.027**
Treatment Duration	0.005	0.965	-0.130	0.224	0.144	0.175	**-0.210**	**0.041**
Final CDIA	**-0.358**	**0.001**	**-0.262**	**0.012**	0.036	0.733	-0.095	0.362
ESR	-0.080	0.456	-0.073	0.492	0.034	0.752	-0.067	0.521

Hb	0.115	0.280	0.191	0.072	0.139	0.192	0.154	0.136
Dose	0.062	0.562	-0.184	0.082	0.159	0.135	-0.188	0.068
PADI4			**0.640**	**<0.001**	**0.302**	**<0.001**	0.137	0.070
IL-17					**0.238**	**0.001**	0.098	0.195
ACCP							-0.025	0.743

Serum levels of IL-17, PADI4 and ACCP in responder and non-responder patients

The median serum level of IL-17 in non- responder patients was (59.16ng/l) which was higher than that of responder one (50.83ng/l) with a significant difference. Also, the median serum level of PADI4 in non- responders was (2.69ng/ml) which was higher than those of responsive (2.4ng/ml) with a significant difference. Regardless of the higher median level for ACCP in non- responder patients no significant differences were seen (Table 8).

The correlation among mi-RNA-499, IL-17, PADI and ACCP with other variables

PADI-4 demonstrated a significant negative correlation with final CDIA (r= -0.358, p=0.001). On the other hand, IL-17 had a significant negative correlation with CDAI (r=-0.262, p=0.012) as shown in (Table7). However, ACCP demonstrated a significant positive correlation with age (r= 0.178, p =0.018), PADI-4 (r= 0.302, p<0.001) and IL-17 (r= 0.328, p=0.001). miR-499a expression was observed to have a significant positive correlation with body weight (r= 0.146, p= 0.042) and BMI (r= 0.159, p= 027) and a significant negative correlation with treatment duration-(r=0.210, p=0.041) (Table 9).

DISCUSSION

The average age of the rheumatoid arthritis patients in our study was 46.22±11.33 years, with the highest rate of RA seen in patients aged 31-45 years followed by the group in the age of 46-50 years. This agrees with a previous study, wherein RA onset was shown to occur between 30-50 years of age, considered a working age in the population (15, 16). Further, RA is found to be more common in women than in men (17). In our results the female-to-male ratio was approximately 3:1. This finding aligns with previous studies (15,18), which demonstrated that among individuals under the age of 50, the incidence of RA in females was 4-5 times higher than in males. However, in individuals aged 60-70, the ratio of males to females was approximately shown to be 1:2 (18). Although the exact reason for the higher prevalence of RA women is not fully understood, several studies have attributed it to the role of sex related genetic factors as well as sex hormones (19). Elevated estrogen and decreased androgen levels have been suggested as an important factor potentially influencing the pathogenesis of RA (18-20).

Among other factors, BMI was found to be significantly associated with RA in this study, which agrees with an earlier study, wherein BMI was shown to significantly increase in overweight and obese patients (21). Smoking has been implicated as one of the most important risk factors for the development and severity of RA (22) which probably explains for the higher frequency of smokers seen among RA patients in this study. The current study also showed that positive family history significantly increased the risk of RA occurrence. According to Frisell *et al.* (23), family history is one of the most potent known risk factors for RA development, with risk of the disease increasing by two to four-fold among first-degree relatives.

MiRNA-499 has been implicated in various diseases, including rheumatoid arthritis. Our work on expression levels for miRNA-499a showed it to be overexpressed in serum of RA patients', which is consistent with the findings of Ayeldeen *et al*., (24), who reported miRNA-146a and miRNA-499 to be significantly upregulated in RA patients, and concluded that these miRNAs could be used as diagnostic markers for rheumatoid arthritis (24). It is important to note, however, that the specific effects of miRNA-499 overexpression in RA are still being studied, and our understanding of its exact role in the disease is evolving. However, enhanced inflammation has been shown to contribute to tissue damage and disease progression. Overexpression of miRNA-499 may disrupt the balance of regulatory T cells (Tregs) and effector T cells, resulting in impaired immune tolerance and the perpetuation of autoimmunity. Furthermore, miRNA-499 functions by binding to target messenger RNAs (mRNAs) and inhibiting or promoting their translation (25). Overexpression of miRNA-499 in RA could lead to dysregulated gene expression patterns, potentially influencing immune response, tissue homeostasis, and other RA pathogenesis-related processes. Other factors, such as the individual's genetic background, disease stage, and the presence of other miRNAs or regulatory molecules, may influence the effects of miRNA-499 overexpression in RA.

Our findings also revealed that RA patients exhibited significantly higher levels of serum IL-17, than healthy individuals which is consistent with a study by Muhammed *et al*., (2014) who reported a significantly higher serum levels of IL-17 and IL-15 in RA patients of Iraq in comparison to individuals with no RA, indicating a direct and strong relationship between these cytokines (26), there are any reports were focused on gene expression of IL-10 as critical biomarkers in rheumatoid arthritis patients (27).

However, the serum levels of PADI4 were significantly elevated in RA patients in this study, which in agreement with studies which have reported elevated serum PADI4 level in RA patients compared to controls (28), and suggested PADI4 as a diagnostic marker for RA (28). Similarly, the serum level of ACCP was higher in RA patients than controls in this study and in line with an earlier report (28). Previous studies have indicated the role of anti-CCP antibody testing as a diagnostic and prognostic tool in rheumatoid arthritis (29).

Citrullinated peptides were detected in the broncho-alveolar lavage fluid of the patients who smoked, and were not present in non-smokers, suggesting that smoking could cause citrullination of peptides in the lung, and that possibly these could promote an immune reaction to citrullinated peptides (29). It's important to note that ACPA testing has become an integral part of the diagnostic criteria for RA, alongside other clinical and laboratory parameters. Detecting ACPA in a patient's serum can help differentiate RA from other forms of arthritis and aid in early diagnosis. With regards to infliximab responsiveness, our findings showed that expression of miRNA-499a is higher in non-responder than in responder patients. However, high levels of miRNA-499 expression in the serum of non-responders may have predictive value for non-responsiveness to infliximab treatment. The current study observed that non-responder patients had significantly higher serum IL-17 levels. This suggests that these individuals produced or released more IL-17 despite being treated with TNF-alpha inhibitors. An elevated IL-17 level in non-responders probably indicates an alternative pathway contributing to ongoing inflammation and pathogenesis of RA disease. Similar findings were obtained for PADI4, which was found to be higher in non-responder patients. This suggests an increased PADI4 activity in non-responder patients, which can result in autoantibody production and contribute to ongoing inflammation and disease activity. Responder patients, on the other hand, had lower PADI4 levels, indicating potentially successful suppression of PADI4 activity and potentially better inflammation. Correlational analysis revealed a statistically significant positive correlation for miRNA-499 expression with BMI and weight, and a significant negative correlation with treatment duration. His implies that miR-499a is possibly associated with storage and distribution of fat in the body, potentially leading to changes in body weight. On the other hand, the negative correlation observed for treatment duration and miRNA-499 overexpression in non-responder patients probably implies the higher expression levels of microRNA-499 to be associated with a reduced response to TNF alpha inhibitors within the initial 6 months of treatment. Finally, a negative correlation between IL-17 and CDAI was also revealed. Regarding ACCP the results of this study revealed that there was a positive correlation between ACCP and each of PADI4 and IL-17.

CONCLUSION

Elevated miRNA-499 expression, along with higher levels of serum IL-17, PADI4, and ACCP, are observed in RA patients compared to controls, suggesting their potential roles in RA pathogenesis. Notably, non-responder patients show significantly elevated levels of these markers, implying their association with treatment un-responsiveness. These findings suggest the promise of miRNA-499, IL-17, PADI4, and ACCP could potentially serve as biomarkers for RA diagnosis, predicting treatment response, and monitoring.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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