MicroRNA-146a Expression and Serum Interleukin-17 Level as potential biomarkers for Rheumatoid Arthritis

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Abstract
Small non-coding RNAs are microRNAs (miRNAs) that can play a role in controlling various immune functions. MicroRNA-146a (miR-146a) is regarded as an essential element in posttranscriptional gene expression regulator, indicating a possible function in autoimmune diseases. Aim: The aim of the research was to evaluate the expression of miR-146a and Interleukin-17 serum levels as potential markers for rheumatoid arthritis (RA) diagnosis and to investigate its association with the activity of the disease. Methods: This research comprised 60 subjects divided into 30 RA patients and 30 healthy individuals. The rate of erythrocyte sedimentation rate (ESR), anti-cyclic-citrullinated peptide (anti-CCP) antibodies rheumatoid factor (RF), C-reactive protein (CRP) and serum IL-17 level were estimated. Using reverse transcriptase real time polymerase chain reaction quantitative, the relative quantification of miR-146a expression was determined. Results: There are highly pronounced statistical variations was observed between patients and healthy controls, with relative expression of miR-146a, (ESR), CRP, IL-17 and (anti-CC). There are also extremely important statistical differences (p < 0.001) between the various patient subgroups with respect to miR-146a relative expression. IL-17 level in the RA group was higher than in the control group. Positive associations were noticed between the levels of IL-17, ESR, CRP, (anti-CCP) and miR-146a. Conclusion: This study showed that even the expression of miR-146a was highly significant in RA patients, the level of expression was associated with the activity of the disease. Also, the increase in serum IL-17 in patients with RA compared with healthy controls played an important role in the diagnosis of the inflammatory and destructive characteristics of RA.

Key words: Rheumatoid arthritis, miR-146a, IL-17, erythrocyte sedimentation rate, C-reactive protein, anti-cyclic-citrullinated.

Introduction
Rheumatoid arthritis, a chronic autoimmune disease, affects %. 0.3-1% of the world's population. It characterized by recurrent inflammation of the joints and structural damage with extra-articular symptoms such as rheumatoid nodules, interstitial pneumonia, vasculitis and systemic complications [1]. Proliferative synovitis occurs in RA, leading to degradation of bones and cartilage [2].

Small microRNAs (miRNAs) are single-stranded endogenous non-coding RNAs that participate in gene expression post-transcriptional regulation, with a length of around 22 nucleotides [3]. The expression of miRNA is correlated with several diseases and has been involved in autoimmune diseases pathogenesis, including rheumatoid arthritis (RA) [4]. RA biomarkers and therapeutic targets have been recommended as miRNA [5]. The number of biological processes were also assisted by miRNAs, for example, apoptosis and the proliferation of cells [6].

MiR-146a responds to stimulation by lipopolysaccharide (LPS) in human monocytes and also its induction is based on nuclear factor κB (NF-κB). MiR-146a is mainly expressed in immune tissues, and its expression can be induced upon maturation and/or activation of immune tissues [7]: [8]. In autoimmune pathogenesis diseases such as rheumatoid arthritis(RA) and erythematous systemic lupus (SLE), MiR-146a plays an important role [9]. MiRNA-146a may regulate the expression of interleukin (IL)-1 receptor-associated kinase (IRAK1), IRAK2 and tumor necrosis factor. Increased miRNA-146a expression level has been found in the RA patients' synovial fluid and peripheral blood mononuclear cells [10]. Cytokines are selectively produced by Th17 cells such as interleukin 17(IL-17), IL-21 and IL-22 have

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been shown to play a critical role in chronic inflammatory response in damaged RA joints and related tissue damage [11]. In addition, IL-23 is a pro-inflammatory kinase that has been found to play a critical role in Th17 cells' differentiation and activation to produce IL-17 in other autoimmune disorders, such as inflammatory bowel disease (IBD) [12].

Interleukin 17 is highly expressed in RA patients' cartilage and nerve fibers. It has been shown that RA synovial fluid stimulates IL-17 and tumor necrosis factor alpha (TNF-alpha) T cells and cytokines, it contributes to the release of IL-6 and IL-8 pro-inflammatory cytokines, which mediate joint inflammation and eventually contribute to cartilage loss [13]; [14].

Aim of the present work: The purpose of this study is to evaluate the expression of microRNA-146a and Interleukin-17 serum levels as potential prognostic markers in the diagnosis of rheumatoid arthritis (RA) and to investigate their relationship with the disease activity

Subjects and methods:
Study design
This observational case–control study was conducted in the period from March to August, 2020.

The work was approved by, National Research Centre Ethics Committee. It was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. Informed consent was obtained from all participants.

Patients and controls
The study involved 60 Egyptian subjects, who were divided into two groups; patients and controls. Patient Group: Included 30 patients recruited from the rheumatology outpatient clinic, National Research Centre. Patients were included in this group after being diagnosed with RA according to the ACR/EULAR classification criteria [15]. Patients suffering from any other autoimmune disorders, chronic illnesses or comorbidities were excluded from the study.

Control Group: Included 30 apparently healthy individuals whose age and sex were matched with those in the patients group.

Data collection
Clinical data were collected in a standardized collection form. Results of clinical rheumatology examination including disease duration, number of tender swollen joints, morning stiffness, extra articular manifestations.

Blood samples were collected from each subject under complete aseptic condition. The samples were divided into two parts; one part was used for analysis of the erythrocyte sedimentation rate (ESR, mm/h), C-reactive protein (CRP, mg/L), IL-17, RF and anti-cyclic citrullinated peptide (anti-CCP). The other part was preserved in tubes containing EDTA at –80 °C for the relative quantification (RQ) of miR-146a expression.

laboratory investigations
RQ of the miR-146a expression
(a) Reverse transcription:
The real-time PCR assay was performed according to the manufacturer’s instructions using a TaqMan PCR package for the analysis of miR-146a (95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds). Relative expression of miR-146a was determined based on the threshold cycle (CT) and was normalized to U6. The data was analyzed using the 2-ΔΔCt process. As follows, the primers of miR-146a and U6 were miR-146a, forward: ACACCTCAGCGTGGTGGAACTGTATCCAT G, reverse: TGTCGTGGAGTCGGCAATTTC; U6, forward: CTGCCTTCGCAAGCACA, reverse: AACGCTTACGAAATTTCGCT. [16].

(b) Quantitative real time PCR:
it was performed following a standard SYBR Green PCR protocol using miScript SYBR Green PCR Kit (Qiagen, Germany) with the StepOne real-time PCR (Applied Biosystems, United States of America). Each reaction mix contained 2× Quantitect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay specific for miR-146a (Qiagen, Germany), template cDNA, and RNase-free water in a total volume of 20 μl. The real-time cycler was programed as PCR was done as follows; enzyme activation at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s annealing at 55 °C for 30 s and extension at 70 °C for 30 s. The expression of the U6B small nuclear RNA (RNU6B) was used as endogenous control for data normalization. The RQ level (fold change) for miR-146a was then calculated using the 2-ΔΔCt method [16].

Biochemical Assays:
Estimation of Erythrocyte sedimentation rate (ESR)
ESR was estimated in mm/1st hour using Westergren method [17].

Estimation of C-reactive protein (CRP)
Serum C-reactive protein (CRP) (CRP–latex slide agglutination test; SPINREAT, S.A.U, Spain [18].

Estimation of Rheumatoid factor (RF)
Rheumatoid factor (RF) was estimated according to nephelometry method, Behring, Marburg, Germany[19].

Quantification of anti-cyclic citrullinated peptide (anti-CCP3)

Anti-CCP3 was measured using ELISA method according to Quanta Lite CCP for IgG/IgA from Enova Diagnostics [20].

Quantification of interleukin-17 (IL-17)

IL-17 was estimated by ELISA kit from Enova Diagnostics according to [21].

Statistical Analysis section:

Using One-way ANOVA, Kruskal-Wallis and Dunn's multiple comparisons, substantial differences between treatments were determined. GraphPad Prism 6.01 was used to conduct all statistical analyses (GraphPad Software Inc., San Diego, CA, USA). P-values below 0.05 were considered significant.

Results

The age for the group of patients ranged from (40-65) years (52.4±7.82) and controls ranged from (40-60) years (52.9±6.69). With respect to age, there was no statistically significant difference between both groups control and patient (Table 1). Significant statistical high differences have been found between patients and controls to miR-146a. IL17, ESR, anti-CCP and CRP (p < 0.0001) (Table 2). It showed that there was a significant difference between the studied RA subgroups, as regards miR-146a and IL-17 with CRB, ESR, CCP and their levels were the highest among those who had severe disease when compared to the others. (Table 3). Also Correlations between miR-146a, RF, anti-CCP and IL-17 had highly significant difference between different groups in patients (Table 4).

The ROC curve was plotted to compare the relative expression efficiency of miR-146a, CRP, CCP, IL17, anti CCP, ESR and RF. MiR-146a, ESR, IL17 and anti CCP illustrated best performance characteristics showing the highest sensitivity and specificity (100% and 100%, respectively) (AUC: 1.000 at a cut off value of P>3.3,>20,>58,>18) (figure1) followed by CRP (sensitivity: 80%, specificity: 96.7% and AUC: 0.933 at a cut off value of P>2.3 and RF (sensitivity:86.7%, specificity:46.7% and AUC: 0.696 at a cut off value of P>24 (figure 2) (Table 5).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group</th>
<th>Patient group</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52.9±6.69</td>
<td>52.4±7.82</td>
<td>0.26</td>
<td>0.79 (NS)</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of the studied participants.

Erythrocyte sedimentation rate; RF, rheumatoid factor; CCP, cyclic citrullinated peptide.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group</th>
<th>Patient group</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a</td>
<td>2.07±0.62</td>
<td>25.14±18.23</td>
<td>6.92</td>
<td>0.000</td>
</tr>
<tr>
<td>ESR</td>
<td>13.73 3.61</td>
<td>54.13±18.03</td>
<td>12.03</td>
<td>0.000</td>
</tr>
<tr>
<td>RF</td>
<td>30.30 ±15.1</td>
<td>46.86± 23.89</td>
<td>3.21</td>
<td>0.002</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>11.53 ±3.96</td>
<td>112.56 ±43.02</td>
<td>12.8</td>
<td>0.000</td>
</tr>
<tr>
<td>CRP</td>
<td>1.35± 0.60</td>
<td>4.66 ±2.33</td>
<td>7.51</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-17</td>
<td>40.96 ±7.6</td>
<td>372.96 ±207.7</td>
<td>8.74</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 2: Comparison between RA patients and healthy controls as regards miR-146a, ESR, RF, anti-CCP and IL-17.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Moderate RF</th>
<th>High RF</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a</td>
<td>10.08 ±3.21</td>
<td>40.2 ±13.88</td>
<td>8.18</td>
<td>0.000</td>
</tr>
<tr>
<td>ESR</td>
<td>39.66± 7.187</td>
<td>68.6 ± 13.16</td>
<td>7.46</td>
<td>0.000</td>
</tr>
<tr>
<td>RF</td>
<td>33.33 ±13.04</td>
<td>60.4 ±24.89</td>
<td>3.73</td>
<td>0.001</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>81.60 ±14.24</td>
<td>143.53 ±39.71</td>
<td>5.68</td>
<td>0.000</td>
</tr>
<tr>
<td>CRP</td>
<td>2.66±0.994</td>
<td>6.66 ±1.31</td>
<td>9.38</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-17</td>
<td>192.26± 35.54</td>
<td>553.66 ±134.87</td>
<td>10.03</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3: Comparison between moderate and high RF as regards miR-146a, ESR, RF, anti-CCP and IL-17.
Table 4: Correlation between miR-146a, ESR, RF, anti-CCP and IL-17.

<table>
<thead>
<tr>
<th></th>
<th>miR-146a</th>
<th>ESR</th>
<th>RF</th>
<th>Anti-CCP</th>
<th>CRP</th>
<th>IL-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a</td>
<td>r</td>
<td>1</td>
<td>.884**</td>
<td>.603**</td>
<td>.884**</td>
<td>.876**</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>ESR</td>
<td>r</td>
<td>0.884**</td>
<td>1</td>
<td>.637**</td>
<td>.944**</td>
<td>.890**</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>RF</td>
<td>r</td>
<td>0.603**</td>
<td>.637**</td>
<td>1</td>
<td>.562**</td>
<td>.686**</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>r</td>
<td>0.884**</td>
<td>.944**</td>
<td>.562**</td>
<td>1</td>
<td>.862**</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>CRP</td>
<td>r</td>
<td>0.876**</td>
<td>.890**</td>
<td>.686**</td>
<td>.862**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-17</td>
<td>r</td>
<td>0.933**</td>
<td>.932**</td>
<td>.609**</td>
<td>.908**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5: Performance characteristics of miR-146a expression, RF, IL-17 and anti-CCP in diagnosing RA.

<table>
<thead>
<tr>
<th></th>
<th>Cut off point</th>
<th>Sensitivity</th>
<th>95% CI</th>
<th>Specificity</th>
<th>95% CI</th>
<th>+PV</th>
<th>95% CI</th>
<th>-PV</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP</td>
<td>&gt;18</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>&gt;2.3</td>
<td>80.00</td>
<td>61.4 - 92.3</td>
<td>96.67</td>
<td>82.8 - 99.9</td>
<td>96.0</td>
<td>77.6 - 99.4</td>
<td>82.9</td>
<td>70.2 - 90.8</td>
</tr>
<tr>
<td>ESR</td>
<td>&gt;20</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>&gt;58</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>miR-46a</td>
<td>&gt;3.3</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.00</td>
<td>88.4 - 100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>RF</td>
<td>&gt;24</td>
<td>86.67</td>
<td>69.3 - 96.2</td>
<td>46.67</td>
<td>28.3 - 65.7</td>
<td>61.9</td>
<td>53.1 - 70.0</td>
<td>77.8</td>
<td>56.5 - 90.4</td>
</tr>
</tbody>
</table>

Discussion:
Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease with varying severity, ranging from moderate to extreme, with both local and systemic manifestations. Phenotypically heterogeneous disease; synovial joints have dangerous inflammatory disorders in their extreme forms, marked by the presence of auto-antibodies in a wide sector of patients [22]. Within damaged joints, high miRNA expressions, such as synovial tissue, PBMCs, plasma, synovial fluid, and activated immune cells have been found in RA patients in various sites and cells. Multiple reports described miR-146a as a significant modulator differentiation and function of inborn and adaptive cells immunities [23]. In the evaluation of RA severity; Blood levels of ESR and CRP have long been the corner stone in distinguishing between the different degrees of RA severity. ESR and CRP, however, lack diagnostic specificity, also ESR and CRP do not generally coincide with or balance the severity of the RA inflammatory phase. In addition, ESR and CRP serum levels do not accurately point to the presence of RA complications. In relation to ESR and CRP, miRNA-146a expression and IL-17 have recently
been introduced to add to the refinement of the ongoing pathology assessment [24]; [25]. In the current study, miRNA-146a expression was measured in RA patients and miRNA-146a overexpression was seen in serum RA patients. These demonstrated that miR-146a may be involved in inflammatory responses in RA disease [26]. We evaluated its expression in RA patients in our research, there were highly statistically significant differences between patients and controls (p < 0.001). The current study agrees with the results of other studies [27]; [28] that have also reported an increase in the expression of miRNA-146a in RA patients. In addition, there was a relation between the expression of miR-146a and disease activity in patients with RA [29].

In comparison, the ROC curve showed that the level of miR-146a was a good diagnostic RA biomarker. This finding was convenient with previous studies [27], that identified elevated miR-146a expression in RA patients in synovial fluid, and whole blood. Our findings showed a substantial positive association between miR-146a levels and disease severity, as shown by the highly significant statistical variation between the expression levels of miR-146a among different subgroups of patients (p<0.001) and the highly significant positive association between miR-146a and CRP (p<0.001, r=0.876), anti-CCP (p<0.001, r=0.884), IL-17 (p<0.001, r=0.933) and ESR (p<0.001, r=0.884). Accordingly, [10] have reported increased expression in patients with active miR-146a in synovium and whole blood RA for elevated activity of the disease.

The performance of miR-146a in the current study was equivalent to that of miR-146a in the diagnosis of RA patients with an AUC of 1,000 folds, RF and anti-CCP with a specificity and sensitivity of 100% and 100%, collectively, at a cut-off value >3.3. [30] recorded that there was an AUC of 0.83 for miR-146a.

In the current study, the mean value of ESR in RA patients was significantly higher relative to the control value (p<0.001) and this is in agreement with [31] who also found that ESR levels was elevated in patients with RA. This can be explained by [32], which indicated that the level of ESR is one of the reactants of the acute phase that is associated with the inflammatory condition such as RA presence. ESR is a basic non-specific inflammatory laboratory measure, however with reduced inflammatory activity, disease damage may progress, and eruptions may develop in patients without clinical symptoms of major inflammation [33]. Increased IL-17 that was detected in peripheral RA blood and synovial fluid is correlated with increased activity of the disease [34]. This may illustrate the effectiveness of serum level in determining RA activity as a predictive item; IL-17 may be used as a particular therapeutic target for RA treatment [14].

These studies were compatible with the [24] who, found that there was a strong positive correlation, among IL-17, ESR, CRP serum levels and miRNA-146a, they showed a significant function for IL-17 serum in the pathogenesis of the destructive and RA-characteristic inflammatory pattern. With frequent attacks, chronic inflammation, there may be mild to moderate changes in CRP expression levels. CRP and ESR are both fine Indicators which represent the state of inflammation RA [35].

Figure1: ROC curve analysis of miR-146a, ESR, anti CCP and IL17 showing the high sensitivity and specificity

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Conclusion
This study showed that the expression of miR-146a was extremely high compared to healthy controls and its level of expression associated with disease activity. Also shows a significant function for IL-17 cells in the pathogenesis of the infectious and damaging pattern characteristic of RA. These two biomarkers could be used in prognosis of RA.

Conflicts of interest
The authors have no Conflicts of interest.

Acknowledgement
Many thanks to all our patients, their parents and all the staff of National Research Centre for their great help during the study.


Figure 2: ROC curve analysis showed RF (AUC=0.696, p=0.004) and CRP (AUC=0.933, p<0.001).