PLASMA microRNA-16 EXPRESSION AS A POTENTIAL BIOMARKER FOR DISEASE ASSESSMENT IN NEWLY DIAGNOSED RHEUMATOID ARTHRITIS PATIENTS

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ABSTRACT
Rheumatoid arthritis is a common chronic autoimmune disease of unknown etiology affecting about 1% of the world population. The synovial tissues in the hands and feet are the main target for the inflammatory process in RA which leads to cartilage and bone destruction and subsequent deformity. It is an essential demand, to distinguish RA cases from non RA at early stages for better prognosis. Thus, through extensive researches microRNAs expression were emerged as promising disease predictors in future. In the current work, we measured and compared the expression of microRNA-16 in the plasma of newly diagnosed RA patients and in healthy controls, and correlated the level of this gene expression with RA associated different clinical and laboratory parameters. With this approach we aimed to assess the potential role of plasma mir-16 as a biomarker in newly diagnosed RA patients and a predictor for disease outcome. Real time polymerase chain reaction analysis was revealed that plasma mir-16 was differentially expressed in RA patients; making it a potential biomarker in RA diagnosis and monitoring disease outcome.

KEYWORDS: rheumatoid arthritis, miRNA, plasma, tender joints, DAS28.

INTRODUCTION:
Rheumatoid arthritis (RA) is a common progressive autoimmune disease of unknown etiology affecting about 1% of adults with 2-3 times more prevalent in females than in males (Rudan et al., 2015). Characterized by long lasting inflammation primarily affects joints rendering them painful, swollen and deformed with limitation in movement, especially wrists, fingers, ankles and feet typically in symmetrical pattern, a characteristic not found in other forms of arthritis (Boman et al., 2017). Although, joint's synovium is the primary target of RA; resulting in subsequent lifelong disability and considerable economic consequences, a variety of extra-articular manifestations may also occur such as, cardiopulmonary disease, glomerulonephritis, and anemia; which may reduce life expectancy up to ten years in severe cases (Abubakar et al., 2015).

Beyond the doubt, identification and treatment of this disease at initial presentation can retard or prevent joint erosions and ultimately altered disease course even to a remission state. However, differentiating RA from other pathologies with similar manifestations at the onset of disease doesn't straightforward till now, because of insufficient clinical and laboratory evidences (Heidari, 2011). Therefore, it is essential to find out suitable parameters for identification of RA patients at early stages and to find predictors of disease outcome (Filiková et al., 2013). Extensive work were guided toward the genetic background of this disease, in which miRNAs had been occupied the central part as the orchestrate molecules in RA pathogenesis (Castro-Villegas et al., 2015). Micro RNAs are endogenous, 22-nucleotide, single-strand, non-coding RNAs that are conserved from worms to mammals and function as modulators of multiple protein-encoding genes at the post-transcriptional level, through binding to the 3'-untranslated regions (3'-UTR) of messenger RNAs (mRNAs), inducing their degradation or inhibition of translation and thus reduce or inhibit protein expression. Identification of miRNAs in the tissues as well as circulating in different body fluids drew further attention to the great potential of these molecules as a disease biomarker (Sohel, 2016). A growing body of confirmation suggests that miRNAs participate in the inflammatory disorders including RA (Mendell & Olson, 2012). Although, in the last few years, great evidences improved that miRNA are promising potential diagnostic biomarkers for RA and a target for new therapeutic strategies. But nonetheless, more extended researches still in need to reach this goal, because a dozen miRNAs' genes were reported to be abnormally expressed in RA, beside, growing evidences that each miRNA regulates several genes, and multiple miRNAs thought to guide a single mRNA over multiple target sites (Churov et al., 2015; Ospelt et al., 2017).

A variety of miRNAs is frequently reported to be dysregulated in different cell types and biological fluids from patients with RA. Among them, miR-16 was reported to target the adenine-uridine-rich elements (AREs) area located in the "3'-untranslated region" of TNF-α mRNA, leading to reduction of this mRNA half-
MicroRNA-16 biomarker for diagnosed rheumatoid arthritis

life and subsequent suppression in the proinflammatory TNF-α production (Pauley et al., 2008; Luo et al., 2010). In this case-control study, we aimed to analyze cell free profile of miRNA-16 in the plasma of newly diagnosed RA patients who had previously received no steroids or any biological treatment, with exception for non-steroidal anti-inflammatory drugs (NSAIDs) as analgesics; also investigated differences in miRNA-16 expression in plasma based on different clinical and laboratory measured parameters in RA patients. With this approach we hoped to identify noninvasive potential biomarkers for early diagnosis and monitoring disease outcome in RA.

MATERIALS & METHODS

Ethical approval for this prospective study was granted by a scientific committee in Baghdad University/college of dentistry as well as Iraqi Ministry of Health. All participants were asked for their permission to give blood samples, and informed consents were obtained for their agreement. Samples collection from patients newly diagnosed with rheumatoid arthritis; attending the rheumatology consulting clinic or admitted to the rheumatology ward (10th floor) in Baghdad Teaching Hospital. Forty newly diagnosed RA patients were included in this study as a case group; all were newly diagnosed as RA patients that formerly did not receive steroids or any immunomodulatory therapy, however, some of them might be treated with "nonsteroidal anti-inflammatory drugs (NSAIDs), and all had a standardized evaluation according to 2010 ACR/EULAR Classification Criteria (Aletaha et al., 2010). Twenty age and gender matched healthy subjects were enrolled as a control group.

Sample collection

According to the manufacturer protocol, venous whole blood from all participants was collected in "BD Vacutainer Venous Blood Collection Tubes" containing EDTA-K3 as anticoagulant; then centrifuged for 10 minutes at 3000 rpm and 4°C with a cold microcentrifuge. After that the upper yellow plasma phase containing cell-free nucleic acid, transferred into a new 2 ml micro centrifuge tube, while the intermediate buffy layer containing platelets and white blood cells shouldn’t be disturbed. Re-centrifuge the sample for another 10 min at 10000 rpm and 4°C, to eliminate additional cellular debris contamination by RNA and genomic DNA (g DNA). Cleared supernatant transferred in aliquots, to avoid repetitive freezing and thawing, and stored at −80°C, until further processing.

Disease Activity Assessment

Each patient had his own case sheet filled with information regarding his/her age, medical history, joints physical signs and ESR test. Each candidate in this group scored for disease activity, according to Disease Activity Score 28 (DAS28) (Fransen et al., 2004), this index consists of a TJC (tender joint count) range 0–28; a SJC (swollen joint count) range 0–28; ESR measurement (as a marker of inflammation) was done according to Westergren’s method; (McCarthy et al., 2014) and a visual analog scale for PGA (patient global assessment) range 0–100. DAS28 calculation was made with online software "http://www.4s-awn.com/DAS28/ DAS28. html" installed in the cell phone; (Faiq & Cabm, 2012).

After DAS28 assessment, RA patients fall into one of the following 3 categories:

- Low, when DAS 28 ≤3.2.
- Moderate, when DAS 28 ≤5.1.
- High, when DAS 28 > 5.1.

RNA extraction

Cell free total RNA including the miRNA fraction, was purified from plasma by using (miRNeasy Serum/ Plasma Kit"; cat.no 217184; Qiagen/ Germany). 200 μl of sample (plasma) was lysed in 5 volumes QIAzol Lysis Reagent then incubated for 5 min at room temperature for RNases inactivation; 3.5 μl synthetic and non-human C. elegans 39 miRNA mimic with a sequence of 5UCACCGGGUGU AAAUCAGCUUG-3 (miRNeasy Serum/Plasma Spike-In Control"; cat. no. 219610; Qiagen/ Germany) was spiked in each sample, in a concentration of 1.6 x 10^6 copies / l, for the purpose of sample-to-sample normalization of the RNA recovery and reverse transcriptase efficiency Kroh et al., 2010). The detailed manufacturer extraction protocol (miRNeasy Serum/Plasma Handbook) can be found at www.qiagen.com/handbooks. Then, RNA concentration and quality was determined with a Nano- drop spectrophotometer (BioDrop/ UK). The final eluted cell free total RNA volume was 14 1 can be stored at −80°C for subsequent processing.

Reverse transcriptase

Purified total RNA containing miRNA, was used as a template for (miScript II RT Kit"; cat. no. 218161; Qiagen/ Germany) to synthesis complementary DNA (cDNA); that was achieved with miScript HiSpec Buffer. In this step, polyadenylation of mature miRNAs by poly-A polymerase, then converted into cDNA with oligo-dT priming by reverse transcriptase.

Real time polymerase transcriptase:

According to manufacturer protocol, cDNA prepared in the previous phase; will serve as a template for mature miRNA quantification, by Stratagene qPCR System (Agilent Technologies/ Germany) together with (miScript SYBR Green PCR Kit"; cat. no. 218073; Qiagen/ Germany); and miScript MicroRNA Assay primer for human miR-16 (Products No. 218300; Qiagen/ Germany). This primer was designed to target and amplify a sequence of (UAGCAGCAGUAAUA UUGGCCG). Cycling condition shown in table 1.

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TABLE 1: Cycling condition for Real-time cycler programming of Stratagene qPCR System, according to the manufacturer protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial activation</td>
<td>15min</td>
<td>95°C</td>
<td>HotStarTaq DNA Polymerase activation</td>
</tr>
<tr>
<td>3-step cycling:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 s</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>30 s</td>
<td>55°C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>30 s</td>
<td>70°C</td>
<td>fluorescence data collection</td>
</tr>
<tr>
<td>Cycle no.</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cycle threshold (Ct) values were determined as well as demonstration of the amplification plots and dissociation curves for each target gene. Ct value represents the number of amplification cycles that was required for the fluorescent signal to overrun the background level; thus, the higher Ct value, the lower amount of the target gene, and vice versa (Westermann et al., 2009). The ∆Ct value of the target gene (Ct target gene - Ct spiked-in reference gene) was obtained to normalize the amount of the used template, in addition to a uniform baseline as well as threshold line settings were performed across all runs in the same analysis, to permit direct comparison of samples. Dissociation curves at the end of each amplification reaction showing a single peak within the range of 76-78°C temperature were detected for each sample, indicating the absence of possible existence of non-specific priming according to the manufacturer protocols.

RESULTS & DISCUSSION

Gender distribution
Out of 40 RA patients, 29 females (72.5%) and 11 males (27.5%); represent a female: male ratio (2.6: 1). This finding coincides with other studies inside Iraq as well as world-wide; although the ratios were variable, but still higher in females when compared to males (Jassim et al., 2015; Al-Herz et al., 2016; Hruskova et al., 2016).

This diverse variances in range of female: male ratio may be in part, due to the inclusion criteria specifically designed for each study; in ours, only treatment-naive RA patients with exception for NSAIDs, regardless their age duration with the disease, and without any other systemic disease or complications were included. Moreover, gonadal hormones, genetic variations, lifestyle and environmental exposure, all were substantially responsible for differences in female: male ratios for RA (Widdifield et al., 2014).

Age distribution
The mean age for RA patients was 43.6 years; allocated into three age groups, as shown in Table 2. The highest incidence for RA in this study was reported in the fourth and the fifth decades which represented 47.7% of the participants, followed by patients of less than 40 years (37.5%) then ≥ 60 years (15%). That was to some extent, similar to what were reported by others like Jassim et al., in which 27.4% of their RA sample aged ≤ 40 years and 72.6% were aged > 40 years (Jassim et al., 2015).

TABLE 2: Patients in RA group distributed according to their ages in three groups

<table>
<thead>
<tr>
<th>Age groups/years</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-39</td>
<td>15 (37.5%)</td>
</tr>
<tr>
<td>40-59</td>
<td>19 (47.5%)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>6 (15%)</td>
</tr>
<tr>
<td>Total</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>

Table 3, represents the reported mean values for the counts of tender (TJC) and swelling (SJC) joints, ESR values and the disease activity scores for RA patients according to DAS28.

TABLE 3: Recorded parameters regarding the count numbers of TJC, SJC, ESR and DAS28 of RA patients

<table>
<thead>
<tr>
<th>TJC</th>
<th>SJC</th>
<th>ESR (mm/h)</th>
<th>DAS28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>6-27</td>
<td>0-22</td>
<td>6-142</td>
</tr>
<tr>
<td>Mean</td>
<td>15.47</td>
<td>7.52</td>
<td>57.03</td>
</tr>
<tr>
<td>SD</td>
<td>6.65</td>
<td>6.59</td>
<td>34.65</td>
</tr>
<tr>
<td>SE</td>
<td>1.05</td>
<td>1.04</td>
<td>5.48</td>
</tr>
</tbody>
</table>

Case-control ∆Ct mean values comparison
The mean ∆Ct value of miR-16 in RA cases (3.127) was higher than that in the control group (2.975), which means it was down regulated, with a statically significant level (p= 0.005) as shown in table 4.
In the current study, mir-16 gene expression was recorded to be down-regulated. A study on early RA that conducted by Filková et al., 2013 but it was on the serum fraction of the blood: reported down-regulation of this gene in RA patients in comparison to established RA group with no difference to controls (Filková et al. 2013). Plasma gene expression of mir-16 in RA patients was studied by Murata et al., but their target was treated RA patients and they reported insignificant difference in this gene expression in comparison to controls (Murata et al., 2010). Additionally, Pauley et al. reported this gene up-regulation in peripheral blood mononuclear cells (PBMCs) of RA patients in comparison to healthy controls and to a disease control group including patients with Sjögren’s syndrome, systemic sclerosis and systemic lupus erythematosus (Pauley et al., 2008).

According to this controversy in findings, it was hypothesized that cell-free circulating miRNAs may not necessarily reflect its intra-cellular levels due to the presence of a cellular- selective mechanism for miRNA release (Pigati et al., 2010). It was supposed that in RA, miR-16 extensively taken-up by specific cells where it applies its regulatory action. Therefore its levels may be modulated by many factors like disease duration, activity and treatments (Pauley et al., 2008).

In this work, although, females were recorded to be more than twice and a half time affected by RA than males. Gender difference was reported to have no effect on gene expression of mir-16 in the plasma (p = 0.31).

In respect to disease activity at the time of diagnoses with RA according to DAS28 index; a notable dominance of patients with high disease activity, 36 cases (90%) was observed in this study, while only 4 (10%) were with moderate activity and no RA candidate reported to have low disease activity at the time of diagnoses; mir-16 was statistically significant (p = 0.01) down regulated in high disease activity RA group in comparison to controls. On the other hand, RA patients with moderate disease activity also showed down regulation in mir-16 expression, but failed to reach statically significant level (p = 0.6). Accordingly, we may postulate plasma as a suitable biological fluid for monitoring disease activity in RA.

Although, cell- free circulating miRNAs gene expression in the sera was out of the scope in this work, Filková et al. were also postulated mir-16 gene expression in the serum as a novel biomarker for monitoring disease activity and response to therapy in RA patients; as they found that a higher level of mir-16 gene expression correlated with a better improvement in arthritic condition based on a larger reduction in DAS28 after establishment of ARDMDs therapy (Filková et al., 2013). Further support, for the importance of mir-16 as a possible biomarker for monitoring RA outcome was by Pauley et al. who reported down-regulation of this gene in peripheral blood mononuclear cells (PBMCs) of treated RA patients with low disease activity in comparison with treated RA patients who having high disease activity (Pauley et al., 2013).

Regarding age time with the symptoms up to their diagnosis as RA patients; only 13 (32.5%) RA patients had disease duration of less than a year, shown insignificant down-regulation in mir-16 gene expression. While the rest 27 (67%) RA patients, had disease duration more than a year and showed statistical significant (p<0.05) down regulation in mir-16 gene expression in comparison to healthy controls. This delay in miRNA-16 gene down-regulation in plasma after starting RA manifestations may postulate that this miRNA gene expression on its way to change its expression to fight against increasing expression of the RA associated inflammatory TN-α.

Linear correlations analysis

In the current work, DAS28 failed to correlate significantly with miRNA 16 genes expression in the plasma (r= 0.28; P=0.07). This finding agreed in partial with a report by Filková et al. on treatment naïve RA patients, but in their sera, as they reported insignificant correlation between mir-16 and DAS28 (Filková et al. 2013). On the other hand, a negative correlation between mir-16 and DAS28 was reported by Murata et al in the plasma of RA patients on different biological treatments (Murata et al., 2010). The only statically significant correlation in this study was reported between mir-16 in plasma and TJC, and it was moderate positive linear correlation (r=0.316; p=0.047) as shown in table 5 and figure 1. While Murata et al., 2010 in their study on RA patients with different biological treatments, reported a negative correlation between TJC and this miRNA gene expression in plasma (Murata et al., 2010).

This significant correlation with TJC beside its general significant down-regulation in RA, may suggest mirRNA-16 as one of the possible potential biomarkers in plasma for monitoring RA outcome. While SJC in this work, was failed to reach a statistical significant correlation with this miRNA in the plasma (r=0.10; p=0.52).

Regarding ESR, this parameter was unable to reach significant level in its correlation with gene expression of mir-16 in plasma (r=0.17; p=0.29). ESR according to Murata et al didn’t show significant correlations with mir-146a and mir-16 genes expression in the plasma, but in RA patient on treatment (Murata et al., 2010). Another support for this result in the current study was by Pauley et al. in which mir-146a and mir-16 gene expression in PBMCs were measured twice over a two-month interval, before and after establishment of MTX treatment,

### Table 4: Case- control differences in means for the ΔCt values of mir-16 in plasma

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>P(t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>40</td>
<td>20</td>
<td>0.005 [S]*</td>
</tr>
<tr>
<td>Range</td>
<td>2.74- 3.50</td>
<td>2.59 -3.39</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.127</td>
<td>2.975</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>1.96</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>0.31</td>
<td>0.40</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05=significant
although ESR values were recorded to be significantly increased despite treatment, while these miRNAs level were unchanged significantly in their RA sample (Pauley et al., 2008).

**TABLE 5: Linear correlation coefficient analysis for ΔCt means values**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28</td>
<td>r= 0.28; P=0.07</td>
</tr>
<tr>
<td>TJC</td>
<td>r=0.31; p=0.04*</td>
</tr>
<tr>
<td>SJC</td>
<td>r=-0.10; p=0.52</td>
</tr>
<tr>
<td>ESR</td>
<td>r=0.17; p=0.29</td>
</tr>
</tbody>
</table>

Correlation is significant at the 0.05 level (2-tailed)

**FIGURE 1:** Scatter diagram and fitted regression line representing a moderate statically significant positive relation between miR-16 in plasma of RA and TJC

**CONCLUSION**

Significant down-regulation of miRNA-16 gene expression in the plasma of RA patients well differentiating RA patients from healthy controls and could be a good noninvasive biological biomarker for characterization of RA patients with highly active disease, and those have disease duration more than a year. As well directly correlated with the number of painful joints in RA patient; may shed light on new treatment strategy of this disease with mir-16 in future.

**REFERENCES**


MicroRNA-16 biomarker for diagnosed rheumatoid arthritis


