Cell Free miRNAs (146a and 26a) Expression in Plasma and Saliva as Potential Biomarkers in Newly Diagnosed Rheumatoid Arthritis Patients

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Abstract: *Rheumatoid arthritis is a common systemic autoimmune disease of uncertain etiology, characterized by chronic inflammation of the synovial tissues and progressive articular damage. Up to date, the most critical issue in rheumatoid arthritis is the need for reliable biomarkers for early diagnosis and monitoring individual responsiveness to therapy. Extensive researches highlight micro RNAs expression as promising disease predictors in future. In the current work, we measured and compared the expression of three selected microRNAs, in plasma and saliva of newly diagnosed RA patients and controls. Real time polymerase chain reaction analysis was reviled that miRNAs (146a and 26a) were differentially expressed in RA patients vs. healthy controls; making them as potential biomarkers in RA diagnosis.*

Keywords: rheumatoid arthritis, miRNA, plasma, saliva

1. Introduction

Rheumatoid arthritis (RA) is a common progressive autoimmune disease with long lasting inflammation primarily affects joints; leads to bone and cartilage destruction, deformity and eventual lifelong disability. Although, joint's synovium is the primary target of RA, a variety of extra-articular manifestations may also occur, like rheumatoid nodules, vasculitis, cardiopulmonary disease, glomerulonephritis, neuropathy and ophthalmologic manifestations, which usually associated with increased morbidity and mortality [1, 2].

From the existing epidemiological data, the worldwide prevalence of RA is about 0.5% - 1% of adults, while, the incidence is believed to be 5 - 50 new cases per 100,000 person each year [3, 4].

Although RA triggers both gender, at any age in all ethnical races, but it tends to occur 2.5 more frequent in women than in man; with peak incidence age within fourth and fifth decades of life; it is more prevalent about 5.3%-6.8% in native American Indian groups [5] and less prevalent among Japan, China and black Caribbean people [6]

Studies have shown that diagnosis and treatment intervention at earlier stage of RA have great impact on disease outcomes even to a remission state. However, recognition of RA from non-RA patients at the early stage of disease is not straightforward, because of inadequate clinical and laboratory evidences [7].

Up to date, numerous predictors of response to different RA therapies like cytokine profiles or gene expression analysis have been reported, but they are either nonspecific or impractical to be adopted in routine clinical practice. Therefore, it is essential to find out suitable parameters for identification of RA patients at early stages and to find predictors of their response to treatment for optimization of individual management based on expected disease course [8]. MicroRNAs (miRNAs) are highly conserved, single-stranded RNA, small molecules about 22 nucleotide bases in length. They regulate genes expression through binding to the 3'-untranslated regions (3'-UTR) of specified messenger RNAs (mRNAs) inducing their degradation or inhibition of translation and thus reduce or inhibit protein expression. This decline in protein expression has been implicated in numerous cellular activities like inflammation, apoptosis, cell cycle regulation, differentiation, and cancer [9]. 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. Moreover, a survey conducted on 12 different body fluids by Weber and her team, reported saliva as the richest body fluid that contained the highest number of detectable miRNAs about 458, while plasma contained 349 types. Also some miRNAs like mir-26a was reported to be uniquely excreted in saliva, however saliva shares large numbers of miRNA species with plasma in compression to other biological fluids which may be due to exchange between them; although, measurably they are different. With the concept of the least invasive technique, saliva could harbor promising and reliable biomarkers for many diseases [9, 10]. A growing number of studies have reported dysregulation of different miRNAs expression contribute many human pathologies like cancer, cardiovascular diseases, neurodegenerative diseases, and autoimmune diseases as RA [11]. MiRNA expression was extensively studied in different RA cell types and body fluids, and reported to have altered expression [12].

In this case-control study, we aimed to analyze cell free profile of tow selected miRNAs (146a and 26a) in plasma and saliva of newly diagnosed RA patients who had received no previous treatment for RA, with exception for non-steroidal anti-inflammatory drugs (NSADs); also compared

plasma miRNA with salivary miRNAs in RA patients in addition to investigate possible correlations of these miRNAs with several RA associated demographical parameters. With this approach we hoped to identify noninvasive potential biomarkers in RA early diagnosis.

2. Materials and Methods

Ethical approval for this prospective study was granted by a scientific committee in Baghdad University/ college of dentistry as well as Ministry of Health in Iraq. All candidates were asked for their permission to give blood and saliva samples with 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" [13]. Twenty age and gender matched healthy subjects were enrolled as a control group.

Sample Collection
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.

All participants were refrained from drinking or eating for about 1 hour prior to saliva collection, and then they asked to rinse their mouth with water 5 minutes before collection. Three ml of whole un- stimulated saliva were collected between 8 am and 12 am, in a polypropylene autoclavable sterile centrifuge tube while it was kept in ice to minimize possible changes in gene expression; to be processed within 30 minutes after collection.; then centrifuged at 4000 rpm for 10 minutes at 4°C with a refrigerated microcentrifuge, supernatant transferred into a new tube for further centrifugation at 10000 rpm for 1 minute at 4°C. Finally the upper clear layer were stored at –80°C in aliquots to avoid repetitive freezing and thawing, till the time of RNA extraction [14].

Cell free total RNA extraction:
Cell free total RNA including the miRNA fraction, was purified from plasma and saliva by using (miRNeasy Serum/ Plasma Kit"; cat.no 217184; Qiagen/ Germany). 200 µl of

sample (plasma or saliva) 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 5UCACCGGGUGUAAAUCAGCUUG-3 (miRNeasy Serum/ Plasma Spike-In Control"; cat. no. 219610; Qiagen/ Germany) was spiked in each sample, in a concentration of 1.6×10^8 copies /µl, for the purpose of sample-to-sample normalization of the RNA recovery and reverse transcriptase efficiency" [15].The detailed manufacturer extraction protocol (mirRNeasy 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 µl can be stored at − 80°C for a year without degradation.

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 conversion 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 primers for human miR-146a and miR-26a (Products No. 218300; Qiagen/ Germany). These primers were designed to target and amplify a sequence of (UGAGAACUGAAUUCCAUGGGUU) and (UUCAAGUAAUCCAGGAUAGGCU) respectively. Cycling condition shown in figure 1.
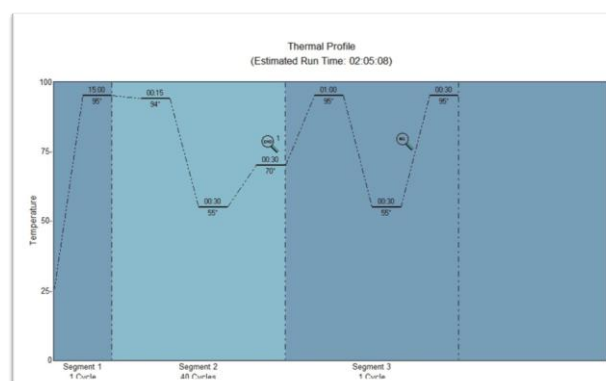
Figure 1: Thermal cycler design (segment 1 and 2) followed in this study based on manufacturer protocol; dissociation curve setting (segment 3) was built in the software of the device

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 is versa. [16]. The ΔCt 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 was performed across all runs in the same analysis, to permit direct comparison of samples. Dissociation curves at the end of an 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.

Statistical analysis:
All data were interpreted in a computerized database structure. "Statistical Package for Social Sciences" (SPSS) version 20 was applied. Comparisons were done using Students t- test and analysis of variance (ANOVA) test; bivariate correlations were done with Pearson correlation analysis; P value was considered statistically significant when < 0.05. The Receiver operating Characteristic (ROC) analysis was done for ranking quantitative parameters in descending manner according to their area under the curve (AUC), to figure out the most affected parameters by the disease status.

3. Results

Out of 40 RA patients, 29 females (72.5%) and 11 males (27.5%); represent a female: male ratio of (2.6: 1).The mean age for them was 43.6 years; allocated into three age groups, as shown in Table 1 and figure 1.

Table 1: Distribution of the RA samples on the age groups

Age groups/years	No. (%)
< 40	15 (37.5%)
40-59	19 (47.5%)
≥ 60	6 (15%)
Total	40 (100%)
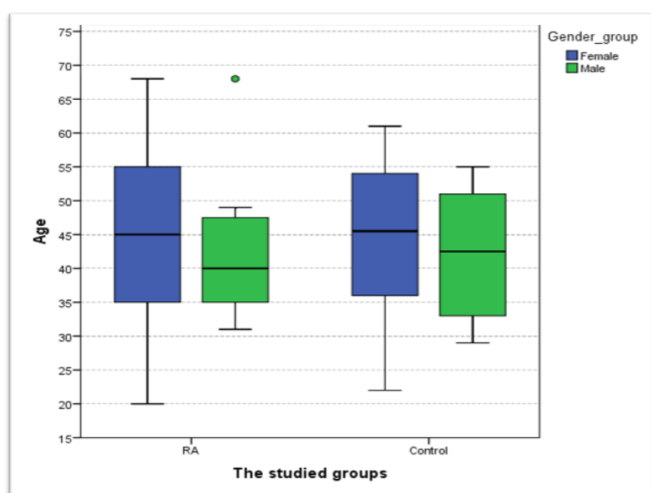
Figure 1: Age distribution of the studied samples

Case-control ΔCt mean values comparisons:
In plasma, The mean raw ΔCt value of miR-146a in RA cases (3.424) was higher than that in the control group (3.302), which means it was down regulated , with a statically significant level (p= 0.019). While the mean raw ΔCt value

of miR-26a in RA candidates (3.168) and in controls (3.081), which failed to reach a statistical level of significance (p= 0.12) as shown in table 2 and figure 2.

Table 2: Case- control differences in means for the raw ΔCt values of the studied micro- RNA in plasma

| Micro RNA	Cases	Controls	P(t-test)
miR-146a			
N	40	20	**0.019[S]***
Range	3.00- 3.79	2.73- 3.70	
Mean	3.424	3.302	
SD	1.57	2.18	
SE	0.25	0.48	
miR-26a			0.12 [NS]
N	38	20	
Range	2.70- 3.54	2.67- 3.59	
Mean	3.168	3.081	
SD	2.02	1.97	
SE	0.32	0.44	

*P<0.05=significant
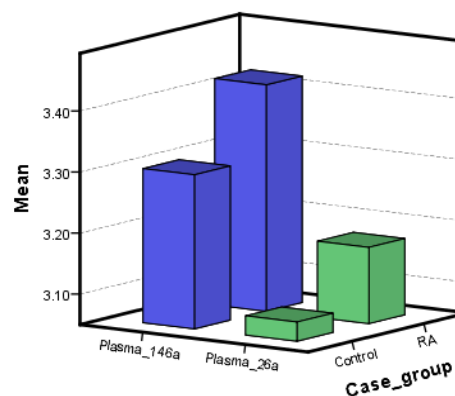
Figure 2: Bar graph showing case-control differences in ΔCt mean values for the micro-RNAs under the study in plasma samples

In saliva: gene expression for the miR-146a was down regulated, as the mean ΔCt value in RA group (3.043) was higher than that in the controls (2.964), but failed to reach a statistically significant level (p= 0.17). While gene expression for miR-26a was up regulated because its ΔCt mean value in RA patients (3.010), was significantly lower (p= 0.04) than that in healthy group (3.145) as shown in table 3 and figure 3.

Table 3: Case- control differences in means for the raw ΔCt values of the studied micro- RNA in saliva.

Micro RNA	Cases	Controls	P(t-test)
miR-146a			
N	40	20	0.17[NS]
Range	2.57 - 3.35	2.52 3.19	
Mean	3.043	2.964	
SD	2.20	1.93	
SE	0.34	0.43	
miR-26a			
N	40	20	**0.04 [S] ***
Range	2.46 - 3.43	2.72 -3.41	
Mean	3.01	3.145	
SD	2.54	1.92	
SE	0.40	0.43	

*P<0.05=significant
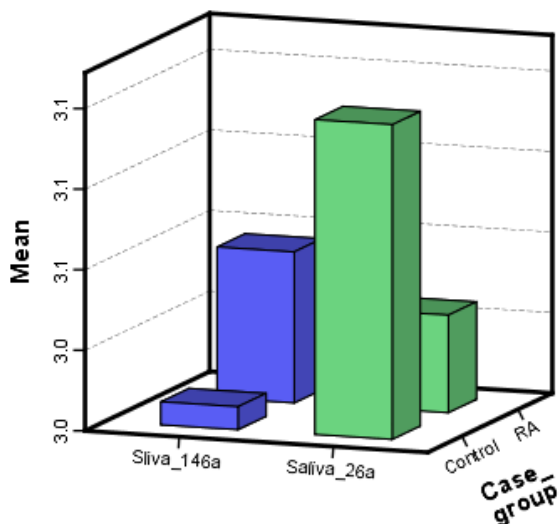
Figure 3: Bar graph showing case-control differences in ΔCt mean values for the micro-RNAs under the study in salivary samples.

Differences in ΔCt mean values in RA stratified by gender:

In plasma, miR-146a gene expression was down-regulated in females compared to RA males but with no significant statistical difference. MiR-26a gene expression was up-regulated in females compared to RA males, but also with no significant statistical difference ($p \geq 0.05$).

In the saliva, miR-146a and miR-26a genes expression were down-regulated, in RA females in comparison to RA male patients, but failed to reach statically significant levels of differences ($p \geq 0.05$).

Linear correlation coefficient analysis for ΔCt means values among the target miRNAs in plasma and saliva samples from RA patients:

Generally, all linear relations in this part of the study were statically insignificant, with exception for mir-146a-1 vs. mir-26a-2 in plasma as they showed moderate positive statically significant relation (r= 0.35; p = 0.03), as shown in table 4 and figure 4.

Table 4: Linear correlation coefficient analysis for ΔCt means values among the target miRNAs in plasma and saliva samples from control candidates

|	mir-146a plasma	mir-26a plasma	146a saliva
mir-26a plasma	**r= 0.35 p=0.03***		
mir-146a saliva	r= -0.15 p= 0.37	r=0.03 p= 0.81	
mir-26a saliva	r= 0.20 p= 0.23	r= 0.03 p= 0.82	r= -0.003 p= 0.98
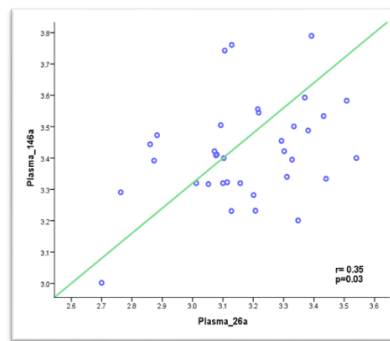
Figure 4: Scatter diagram and fitted regression line representing a moderate statically significant positive relation between miR-146a and miR-26a in plasma.

Receiver operating characteristic (ROC) and area under the curve (AUC) of the target miRNAs was used to rank the studied miRNAs according to the magnitude of their affection by RA. Mir-146a in plasma was ranked first to be the most affected parameter by RA in this study, having statically significant (p=0.037) the highest ROC area (0.67) with a sensitivity of (60%) and specificity (65%) at cut off point of (≥ 3.39); Coming next mir-26a in saliva with statically significant (p=0.034), ROC area (0.67), sensitivity of (63%) and specificity (60%) at cut off point of (≥ 3.095). mir-26a in plasma showed reasonably accepted ROC areas (0.638), but failed to reach statically significant level (p > 0.05), as shown in table 5 and figures 5 and 6.

**Table 5:** Linear correlation coefficient analysis for ΔCt means values among the target miRNAs in plasma and saliva samples from RA patients

ΔCt mean values	AUC	P	
mir-146a/ plasma	0.67	0.037	[S]*
mir-26a / saliva	0.67	0.034	[S]*
mir-26a / plasma	0.64	0.086	[NS]
mir-146a/ saliva	0.36	0.085	[NS]

*P<0.05 = significant.
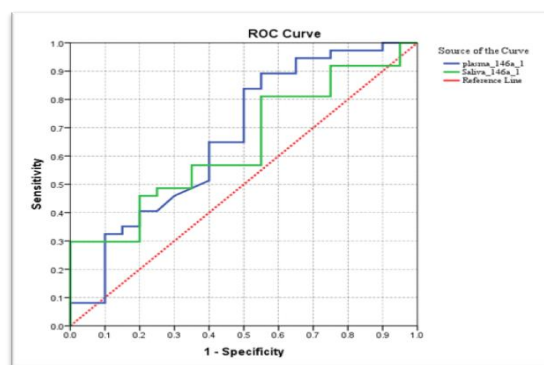
Figure 5: ROC presenting compression of AUC for mir-146a in plasma and mir-146a in saliva in the context of differentiation between cases with RA and healthy controls
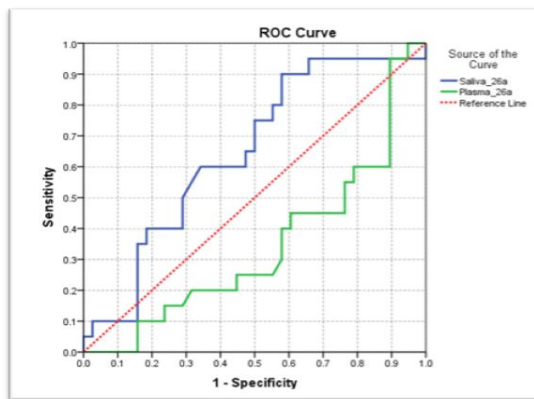
Figure 6: ROC presenting compression of AUC for mir-26a in plasma and mir-26a in saliva in the context of differentiation between cases with RA and healthy controls

4. Discussion

MicroRNAs as biological diagnostic tools have many advantages and challenges. In respect to sample accessibility, although they are stable, in noninvasive different body samples like blood and saliva and can be simply analyzed by widespread "Real-Time PCR" technique, but still their extraction and detection are a matter of challenge due to their relatively low amount beside data analysis for different diseases are in need of more appropriate references. Meanwhile, from the diagnostic point of view, several miRNAs have shown a potential diagnostic ability for early diagnosis of several diseases with increased diagnostic accuracy when several miRNAs are combined, as well as promising predictors in response to therapy, but the challenge is the need for more studies to discover each disease -specific miRNAs [12].

Although, females were recorded to be more affected by RA than males, gender difference was reported to have no effect on gene expression of the 2 miRNAs under the study (146a and 26a), in the plasma as well in the saliva of patients newly diagnosed with RA; suggested the presence of other molecular mechanism other than miRNAs (146a and 26a) orchestrate this gender- biased in RA. And that was in agreement with [17].

MicroRNA-146a:
Significant down-regulation of microRNA-146a in plasma was in part, coincides with a reported down regulation of this gene by [18] on treatment- naïve RA patients, compared to healthy controls, but their studied samples were serum not plasma. Another study was conducted by **[19],** who reported this miRNA down-regulation by miRNA microarray analysis and "real-time quantitative PCR (qRT-PCR)" in plasma of 25 patients, but with established RA compared to 20 healthy controls.

In saliva, and in respect to this work, although a tendency toward under-expression of this gene was reported, but it failed to reach a significant statistical level. These different outcomes between plasma and salivary levels in this gene expression could be due to the difference in their cellular compositions, beside a fact that extra- cellular releases of miRNAs were under the control of cellular selective mechanisms [8]**.**

Considering several studies reported cellular miR-146a up-regulation, in T cells from PBMCs and from synovial fluid (SF) of treatment - naïve RA patients and in fibroblast-like synoviocytes (FLSs). May be suggested that this miRNA could be extensively up-taken- by these cells where it exerts its activity in RA, when compared to healthy controls or osteoarthritis patients [20, 21, 22, 23].

This miRNA is the key molecule in the negative regulation of IL-1β and TNFα inflammatory cytokines; through targeting "IL-1 receptor-associated kinase 1 (IRAK-1) and tumor necrosis factor receptor-associated factor 6 (TRAF6)" [24].This controversy with this finding may explained according to [25], that miRNA down-regulation in the plasma with its propensity toward down-regulation, although insignificant in saliva; might allow the fine regulated system - when corrupted with a pathological condition like RA- to enhance translation of a set of proteins that are minimally expressed in normal situation.

MicroRNA-26a:
This miRNA selection based on former reports; demonstrating its differential expression in RA and underscored its role in regulating TNF- α and IFN-β inflammatory cytokines; which lead inflammatory process and joints damage in RA **[26, 27].**

In saliva, up-regulation of microRNA-26a gene expression was recorded in newly diagnosed RA patients compared to healthy controls. This miRNA over expression could be a result for increased inflammatory cytokines, to stabilize TNF-α mRNA through targeting its "3'UTR **[28].** Although, cell free circulating mir-26a was the target of this study; however, up regulation of this gene in the PBMCs of RA patients was reported by [29].

Our finding may be supported by others' studies on synovial cells and macrophages which reflect abnormal TLR3 gene expression, and/or abnormality in its molecular signaling pathway; both share crucial parts in RA pathogenesis at its early stages. MiRNA-26a orchestrates this scenario at two levels; modulation of TLR3 gene expression through direct interaction of miR-26a with tlr3 mRNA at its 3′UTR, or targeting TLR3s themselves and their signaling molecules to interfere with the activated signaling cascade and control "IFN regulatory factors (IRFs) 3 and 7, resulting in subsequent suppression of TNF- α and IFN-β inflammatory cytokines [27].

5. Conclusions

Plasma miRNAs (146a and 26a) gene expressions have distinct profile from salivary miRNAs. Differential expression of miRNA-146a gene expression in the plasma and miRNA-26a gene expression in the saliva of RA patients; ordered in respect to the magnitude of their affection by RA; Are well differentiating RA patients from healthy controls, and may characterize the early stages of this disease.

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