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MIR-146A AND TLR4 GENE EXPRESSION IN PREDICTING RHEUMATOID ARTHRITIS DISEASE

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ABSTRACT

Disease Activity Score 28 (DAS28) isthe most widely used method for rheumatoid arthritis(RA) disease activity evaluation. However, it may not reflect disease activity accurately being a subjective tool dependent on patient's self-health assessment and evaluation of swollen and tender joint counts. Therefore, finding a suitable blood genetic marker for accurate monitoring of RA disease activity has become essential. Therefore, the aim of this study was to assess miR-146a and TLR4 expression in PBMCs of RA patients and to study their value as potential molecular biomarkers for RA disease activity in comparison to DAS28 score. This study was conducted on 51 RA patients and 15 age and sex matched healthy subjects as control group. The relative gene expression levels of miR-146a and TLR4 were determined by reverse transcriptase quantitative real time polymerase chain reaction. There were statistically significant differences between patients and healthy controls as regards miR-146a and TLR4 expression. In addition, statistically significant differences were observed between different patients' subgroups as regards miR-146a and TLR4 expression. Moreover, miR-146a and TLR4 expression levels showed significant positive correlationswith those of morning stiffness durations, tender joints counts, swollen joints counts, visual analogue scale values, erythrocyte sedimentation rates, CRP, Anti-CCP antibody and DAS28.MiR-146aillustrated the best performance

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characteristics particularly in differentiating between high and moderate disease activity grades, showing highest sensitivity and specificity.Furthermore, there was a statistically significant increase in the expression levels of miR-146a and TLR4 in PBMCs of RA patients with ankles and/or feet joints involvement, as compared with those without involvement.**Conclusion:** MiR-146a and TLR4 were overexpressed in PBMCs of RA patients and were correlated with disease activity and radiographic progression especially miR-146a.

***Keywords*:** DAS28**,** Larsen score, RA**,** reverse transcriptase quantitative real time PCR.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune systemic disorder,which is characterized by chronic inflammation of synovial tissue, causing irreversible damage of joints**(Pauley et al., 2009)**.

Developing a reliable method for assessment of RA progression, and administered therapy effectiveness, is time-consuming. Suggested methods often yield debatable results (van Riel, 1992). Although, there is no real “gold standard” available to judge RA disease activity **(Farnsen et al., 2003)**, the most commonly used method for evaluation of RA activity is the Disease Activity Score DAS28 because ofthe relative ease of its acquisition and calculation. The formulas of DAS28 calculation incorporate the number of swollen and tender joints, self-health assessment by using the visual-analog scale (VAS), and erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) **(Barczyńska et al., 2015)**.

The DAS28 may not provide accurate reflection of disease activity because ofmultiple limitations. One of these limitations is the great weight of the count of swollen joints in the score, as this parameter may not be precisely determined by physical examination provided by a health-care provider. Furthermore, the DAS28 may remain increasedbecause of irreversible damage of joints, which remain tender in spite of subsidence of the inflammatory process **(Barczyńska et al., 2015)**. In addition, ankles and feet joints are not included in the DAS28, which may result in misclassification regarding low disease activity or remission. Furthermore, concomitant fibromyalgia may lead to high DAS28, because of positive association

of tender points with the VAS global and tender joint (TJ) scores. This too might result in misclassification **(Jacobs et al., 2014)**.

Another limitation is the ESR use in the DAS28, since the ESR parameter is age and gender dependent (it rises with age and is elevated in females) and is also dependent on blood cell count (it rises with anemia).This may result in inaccurate reflection of disease activity(Jurado, 2001). CRP can be used in the DAS28 instead of ESR. It is less influenced by other conditions than ESR. However, CRP possess the same drawback as ESR**(Jacobs et al., 2014)**.

Based on the issues described above, applying the treat-to-target principle in a RA patient needsaccurate and valid measurement of the activity of the disease, which has not been achieved completely with DAS28. This has prompted us to search for blood genetic markers for accurate monitoring of RA disease activity and to assist clinical decision-making.

Toll-like receptors (TLRs), a family of conserved pattern recognition receptors, play crucial roles in the innate and adaptive immune systems, and are principally expressed on cells, such as dendritic cells and macrophages.These cells act as primary sensors that recognizeexogenous and endogenous stimuli **(Kawai and Akira, 2007).**Over the past decade, TLRs have been proposed to drive inflammation in RA**(Fui and Kim, 2012)**. Among the family of TLRs, TLR4 can recognize lipopolysaccharide (LPS) which is an integral component of Gram-negative bacteria outer membranes**(Akira and Takeda, 2004).** Upon ligand binding, TLR4-mediatedsignals are stimulated by toll-interleukin-1 receptor domain-containing adaptor inducing IFN-γ (TRIF) and myeloid differentiation factor 88 (MyD88)**(Takeda et al., 2003** and **Lu et al., 2008)** These interactions induce a cascade of intra-cellular signaling including TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1(IRAK1) that results in nuclear factor kappa B (NF-κB) activation. NF-κB activation induces the transcription of pro-inflammatory cytokines and interferons (IFNs) which initiate an inflammatory response **(Saba et al., 2014)**.

MicroRNAs (miRNAs) are non-coding small RNAs that, by base pairing to messenger RNA (mRNA) mediate mRNA cleavage, translational repression or mRNA destabilization **(Filková et al., 2012)**. This mode of posttranscriptional regulation of gene expression has been recently found to play a role in modulating the TLR response

in a broad range of human immune cells, including monocytes, macrophages, and T cells **(O'Neill et al., 2011).**Certain miRNAs were found to be key regulators of development of immune cell and innate/adaptive immune responses **(Pauley et al., 2009)**. Of these miRNAs, miR-146a, a member of the miR-146 miRNA family, emerged as a negative master regulator of TLR activation **(Taganov et al., 2006).** MiR-146a was revealedto be a key regulator of the TLR4-MyD88 pathway by directly targeting IRAK1 and TRAF6 mRNAs (**Boldin et al., 2011andZhao et al., 2011)** and hence, it can dampen down and switch off the inflammatory response to prevent over stimulation of the signal **(Quinn and O'Neill, 2011)** suggesting its role as a "brake on immunity"**(Boldin et al., 2011 and Zhao et al., 2011).**

The aim of this study was to assess miR-146a and TLR4 expression in PBMCs of RA patients and to study their value as potential molecular biomarkers for RA disease activity in comparison to DAS28 score, considered to be the most widely used method for evaluation of RA activity.

MATERIALS AND METHODS

Study participants

Fifty-one RA patients were enrolled in this study, with inclusion criteria of fulfilling American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria for RA(**Aletahaet al., 2010**). The patients were selected during regular follow up at the outpatients' clinic of tertiary referring hospital. Exclusion criteria included Patients not fulfilling the ACR criteria for the diagnosis of RA or had another rheumatic disease, patients with age < 18 years and patients with any other autoimmune disorders or chronic diseases were excluded from this study.Fifteen age-and sex-matched apparently healthy volunteers were also recruited as a control group.

All patients were subjected to full history taking, thorough clinical examination and assessment of disease activity by disease activity score of joint count (DAS28) **(Prevo et al.,1995)**. Disease duration, morning stiffness duration, self-assessment of health measured by VAS, presence of rheumatoid factor (RF), anti-cyclic citrullinated

peptide (anti-CCP) and hemoglobin concentrationswere recorded. All patients were also subjected to radiological assessment through Plain x rays of both hands and wrists. Posterioanterior and lateral views were performed and graded according toLarsen score(**Larsen et al., 1977**). In addition, assessment of the status of ankle and feet joints together with presence or absence of concomitant fibromyalgia was carried out.The patients group was further subdivided according to DAS28 score into: low-(DAS28≤3.2), moderate-(3.2<DAS28≤5.1), and high-disease activity (DAS28>5.1) **(van Gestel et al., 1998)**. All participants gave their written consent after being informed about the purpose of the study, which was previously approved by the Research Ethical Committee.

Blood sampling and processing

Blood samples (5 ml per subject) were collected by peripheral venipuncture**.** The collected blood samples were divided into 2 parts. First part (3 ml) was left to clot for serum separation to be used for laboratory investigations and thesecond part (2ml) was collected in sterile EDTA-treated tubes to be used for peripheral blood mononuclear cells **(**PBMCs) separation. PBMCs were separated by Ficoll density-gradient centrifugation method **(Amos and Pool, 1976)**using FicollHistopaque®-1077 (Sigma-Aldrich, U.S.A.).PBMCs werestored at -80 °C until later assessment of **miR-146a** and **TLR4** gene expression.

Total RNA extraction

Total RNA including miRNAs was extracted using miRNeasy mini kit (Qiagen,Germany) in accordance with the manufacturer's protocol. Purity and concentration of the extracted RNA were assessed by Nanodrop 2000 (Thermofisher scientific, USA).

cDNA synthesis

Extracted RNA (100 ng) was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions.Random primers were used for TLR4 and GAPDH reverse transcription, whereas specific reverse transcription (RT) primers were used for miR-146a and the housekeepingsnRNA U6. Stem loop primer for miR-146a andU6 RT

primer are shown in Table 1. Reverse transcription was carried out on T100 thermal cycler, Bio-Rad, Singapore.

Quantitative real-time PCR:

Real-time PCR was performed using QuantiTect® SYBR® Green PCR (Qiagen, Germany)on StepOne™ (Applied Biosystems, USA).The sequences of the primers used are shown in Table 1.Each reaction contained 10 µl of 2 × QuantiTect® SYBR® Green PCR, 0.7 µl of 10 µM of each primer, 1 µl of the cDNA, to a total volume of 20 µl. The optimized thermal profile included an initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 63°C for miR-146a,66°C for U6,61°C for TLR4 and 70°C for GAPDHand extension at 72 °C for 30 s.Subsequently, at the end of the PCR cycles, specificities of the amplified products were assessed by melting curve analysis. Relative expression of miR-146a and TLR4 mRNA in each sample were finally detected after normalization tosnRNA U6 and GAPDHexpression respectively and calculated as $2^{\wedge-\Delta Ct}$**(Lin et al., 2003),**ΔCt was calculated by subtracting the Ct of the housekeeping gene from that of the target gene. Lower ΔCt values and higher $2^{\wedge-\Delta Ct}$ indicated higher expression level of the target gene.

Table (1): Primers of the genes included in the study

Gene	Primers		Reference
hsa-miR-146a	Stem loop primer	GTCGTATCCAGTGCGTGTCGTGGA GTCGGCAATTGCACTGGATACGACa accca	Xie et al., 2013
	Forward primer	GGGTGAGAACTGAATTCCA	
	Reverse primer	CAGTGCGTGTCGTGGAGT	
TLR4	Forward primer	CTTATAAGTGTCTGAACTCCC	Szebeni et al., 2007
	Reverse primer	TAC CAGCACGACTGCTCAG	
U6	RT primer	CGCTTCACGAATTTGCGTGTCAT	Xie et al., 2013
	Forward primer	GCTTCGGCAGCACATATACTAAAAT	
	Reverse primer	CGCTTCACGAATTTGCGTGTCAT	
GAPDH	Forward primer	TGCACCACCAACTGCTTAGC	Cicinnati et al., 2008
	Reverse primer	GGCATGGACTGTGGTCATGAG	

Statistical Analysis

The collected data were computed and statistically analyzed using Statistical Package for the Social Science (SPSS) version 23 software. Quantitative variables were expressed as mean ± SD or mean±SE. Parametric data were compared using student t- test. Non parametric

data were compared by Mann-Whitney U-test. Kruskal-Wallis test was used for comparison between three groups of not normally distributed variables. Qualitative variables were expressed as number and percentage and were compared by Fisher's exact test. Spearmen correlations were applied for correlating different variables. ROC curve was used to predict the best cutoff values of $2^{\wedge-\Delta Ct}$ of the expressed miR-146a and TLR4 with the optimum sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for prediction of RA disease activity.In all tests, p value less than 0.05 was considered significant.

RESULTS

The RA patients and controls were matched for age (p>0.05) and sex (p>0.05). The age ranged from 30 to 58 years with mean±SD of 41.61±6.45 years in the RA patients and from 30 to 58 years with mean±SD of 42.8±7.56 in the control group. The RA patients group comprised 42(82.4%) females and 9(17.6%) males whereas the control group comprised 12(80.0%) females and 3(20.0%) males. The clinical and laboratory characteristics of the RA patients are shown in Table 2.

Both miR-146a andTLR4expression levels were significantly higher in RA patients than in the control group as shown in Table3.

Kruskal-Wallis test was used to compare between the patients' subgroups (divided according to DAS28) regarding **miR-146a** and **TLR4** expression levels. Highly statistically significant differences ($p < 0.001$) were observed between different patients' subgroups beinghighest in RA patients with the most severe disease activity as shown in Table 4.

In addition, there were highly statistically significant differences as regards both **miR-146a** and **TLR4** expression levels among the different grades of the Larsen score (p< 0.001).**MiR-146a** expression levels were highest in RA patients at grade 1and lowest at grade 5whereas **TLR4** expression levels were highest in RA patients with radiological damage at grade 5 and lowest at grade 1 as shown in Table 4.

MiR-146aexpression levels showed significant positive correlations with those of morning stiffness durations (rho=0.62 and p=0.001), tender joints counts (TJC)(rho=0.82 and p=0.001), swollen joints

counts (SJC) (rho=0.67 and p=0.001), VAS values (rho=0.54 and p=0.001), ESR (rho=0.36 and p=0.01), CRP (rho=0.37 and p=0.008), Anti-CCP antibody (rho=0.53 and p=0.001) and DAS28 (rho=0.61 and p=0.001). However, **miR-146a** expression levels showed no statistically significant correlation with any of theRFtitres (rho=0.15 and p=0.29), age (rho=0.05 and p=0.74), disease durations (rho=0.10 and p=0.48).

TLR4 expression levels showed significant positive correlations with those of morning stiffness durations (rho=0.56 and p=0.001), tender joints counts (TJC)(rho=0.87 and p=0.001), swollen joints counts (SJC) (rho=0.77 and p=0.001), VAS values (rho=0.55 and p=0.001), ESR (rho=0.4 and p=0.004), CRP (rho=0.45 and p=0.001), Anti-CCP antibody (rho=0.66 and p=0.001) and DAS28 (rho=0.70 and p=0.001). On the other hand, **TLR4** expression levels showed no statistically significant correlation with any of the age (rho=0.09 and p=0.53) or disease durations (rho=0.15 and p=0.29). However, there was statistically significant negative correlation between **miR-146aand TLR4** expression levels(rho=-0.89 and p=0.001).

ROC curve was plotted to compare the performance of **miR-146aand TLR4** expression in differentiating between different grades of DAS28 score**.** The best cut off values for 2^-ΔCt of the expressed **miR-146aand TLR4**with the highest, sensitivity, specificity, PPV and NPV were determined and analyzed as shown in Table 5, Figure 1.

MiR-146aillustrated the best performance characteristics particularly in differentiating between high and moderatedisease activity grades showing highest sensitivity and specificity (100%)(AUC: 1.0 at a cut off value of ⩾0.17). On the other hand,**TLR4**performance characteristicswas incomparable with that of **MiR-146a.**It illustrated lower specificities in all gradesand it couldn't differentiate between low and moderate disease activity grades showingthe same cut off value of ⩾0.09.

Changes in TLR4 and miR-146a according to the presence or absence of ankles &/or feet joints involvement, irreversibly damaged joints and concomitant fibromyalgia are represented in Table 6.There was a statistically significant increase in the expression level of miR-146a and TLR4 in PBMCs of RA patients with ankles and/or feet joints involvement, as compared with those without involvement. In addition, there were no significant differences between RA patients with irreversible damaged joints and those without regarding miR-

146a and TLR4 expression levels. Furthermore, no statistically significant differences were observed between RA patients with concomitant fibromyalgia and those without as regards miR-146a expression levels.

Table (2):Clinical and laboratory characteristics of the RA patients

Parameter	Mean ±SE	Reference value
Disease duration (years)	3.29±0.36	-
Morning stiffness (minutes)	79.8±7.03	-
Number of tender joints	7.65±0.54	-
Number of swollen joints	6.04±0.33	-
VAS	5.06±0.34	-
DAS28	5.19±1.41	-
Hemoglobin concentration (g/dL) Male Female	10.59±0.12 10.46±0.99 10.62±0.83	14.0-17.5 12.3-15.3
ESR (mm/h)	57.29±2.24	Male 0-22 Female 0-29
RF (U/ml)	76.0±8.14	< 15
CRP (mg/dl)	14.82±2.29	< 8
Anti CCP (U/ml)	42.54±19.15	< 20
Parameter	Number (%)	
DAS28		
Low activity	9 (17.6)	
Moderate activity	10 (19.6)	
High activity	32 (62.7)	
Larsen score		
Grade 1	12 (23.5)	
Grade 2	12 (23.5)	
Grade 3 Grade 4	15 (29.4) 1 (2.0)	
Grade 5	11 (21.6)	
Involvement ofankles or feet joints	29 (56.9)	
Presence of irreversibly damaged joints	11(21.6)	
Concomitant fibromyalgia	13 (25.5)	

Table (3): MiR-146a and TLR4 normalized gene expression levels in the different studied groups(2^-∆Ct)

Parameter	miR-146a		TLR4	
	Mean ±SE	P value	Mean ±SE	P value
Patients group Control group	0.96±0.21 0.03±0.01	0.001*	2.26±0.39 0.58±0.17	0.004*

*P<0.05 is significant

Table (4): Changes in miR-146a andTLR4according to sex,DAS28 and Larsen scores

Parameter	miR-146a		TLR4	
	Mean ±SE	P value	Mean ±SE	P value
Sex Male Female	1.36±0.76 0.87±0.19	0.71	3.12±1.31 2.07±0.39	0.77
DAS 28 Low activity Moderate activity High activity	0.22±0.06 0.19±0.06 1.42±0.30	0.001*	0.40±0.13 0.60±0.26 3.30±0.54	0.001*
Larsen score Grade 1 Grade 2 Grade 3 Grade 4 Grade 5	2.03±0.51 1.28±0.56 0.48±0.09 0.25±- 0.11±0.03	0.001*	0.23±0.06 1.16±0.35 3.19±0.95 3.64±- 4.68±0.84	0.001*

*P<0.05 is significant

Table (5): Performance characteristics of miR-146a and TLR4 expression (2^-ΔCt) in differentiating between different grades of DAS28

Parameter	Cut off point	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy	P value
miR146a expression level								
At low grade of DAS28	≥0.05	0.97	100	86.7	81.8	100	91.7	0.001*
At moderate grade of DAS28	≥0.11	0.90	100	60.0	62.5	100	76.0	0.008*
At high grade of DAS28	≥0.17	1.0	100	100	100	100	100	0.001*
TLR4 expression level								
At low grade of DAS28	≥0.09	0.56	100	46.7	52.9	100	66.7	0.049*
At moderate grade of DAS28	≥0.09	0.64	100	46.7	55.6	100	68.0	0.037*
At high grade of DAS28	≥0.45	0.84	100	66.7	86.5	100	89.4	0.001*

*P<0.05 is significant

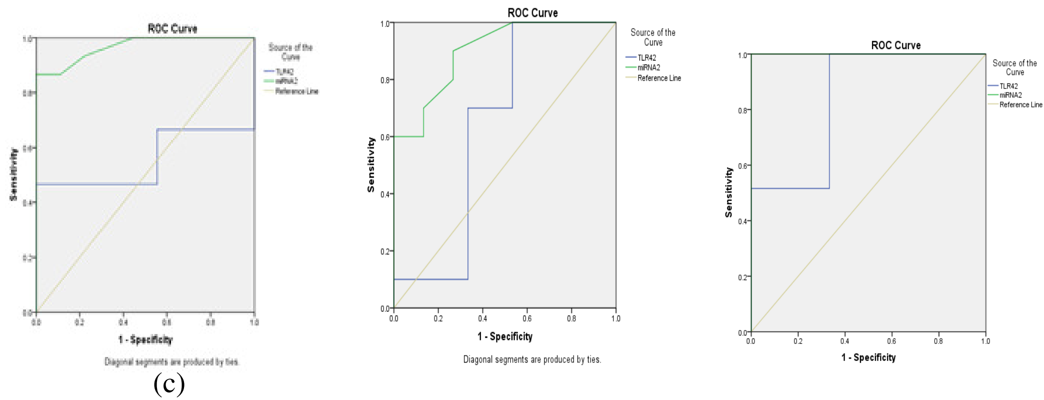

Figure (1):ROC curve of miRNA 146a and TLR4 at different grades of DAS28;a: low, b: moderate, c: high.

Table (6): Changes in miR-146a and TLR4according to the presence or absence of ankles &/or feet joints involvement, irreversibly damaged joints and concomitant fibromyalgia

Parameter	miR-146a		TLR4	
	Mean ±SE	P value	Mean ±SE	P value
Ankles &/or feet joints Involvement • Present • Absent	1.52±0.32 0.18±0.03	0.001**	3.68±0.55 0.38±0.05	0.001**
irreversibly damaged joints • Present • Absent	0.62±0.05 1.05±0.26	0.15	2.38±0.49 1.82±0.35	0.11
Concomitant fibromyalgia • Present • Absent	1.134±0.26 0.41±0.06	0.495	2.73±0.50 0.89±0.18	0.294

*P<0.05 is significant

DISCUSSION

Changes in miR-146a expression have been reported in different human diseases, such as autoimmune and inflammatory diseases, sepsis, viral infections, cancer, and multiorgan failure **(Li et al., 2010)**.

Regarding miR-146a expression levels, the results of the present study showed a statistically significant increase in the expression levels of miR-146a in PBMCs of RA patients, as compared with healthy controls. Several previous studies confirmed upregulation of miR-146a in PBMCs of RA patients **(Pauley et al., 2008, Niimoto et al., 2010 and Murata et al., 2010)**. Furthermore, the results of the current study were in concordance with **Nakasa et al.(2008)** and **Stancyk et al.(2008)** who stated that miR-146a was highly expressed in RA synovial tissue and fibroblasts and its expression mimics that of PBMCs.

Detection miR-146a in circulating PBMCs of RA patients opened the field for its utilization as a biomarker for monitoring the course of the

disease and treatment efficacy without needing surgical intervention to get joint tissues to analyze miRNA.

Upregulation of miR-146a in RA patients could be explained by the fact that transcription of miR-146a is induced through the NF-κB-dependent pathway in response to various pro-inflammatory immune mediators, such as LPS, IL-1β, TNF α and latent membrane protein 1 (LMP1), which are increased in RA patients **(Taganov et al., 2006)**.

Regarding miR-146a expression levels in autoimmune diseases and other diseases, increased expression of miR-146a in skin lesions and peripheral blood of psoriasis patients was previously reported **(Sonkoly et al., 2007 and Azab et al., 2017)**. Upregulation of miR-146a in psoriasis patients limits its use as a specific diagnostic marker for RA. On the contrary, **Tang et al. (2009)** showed a 3-fold down regulation of miR-146a in systemic lupus erythromatosis (SLE) patients. Also **Balasubramanyam et al. (2011)** reported that miR-146a expression levels decreased significantly in PBMCs of Type 2 diabetic patients.

The controversial observations on miR-146a expression levels in different autoimmune diseases could be attributed to various pathogenic pathways in which miR-146a is involved in different diseases.

The result of the present study showed that miR-146a expression was positively correlated with the morning stiffness durations, VAS score, ESR, CRP, TJC, SJC, anti-CCP and DAS28. Moreover, highly statistically significant differences were observed between different patients' subgroups as regards miR-146a expression, beinghighest in RA patients with the most severe disease activity and lowest in those with low-grade disease activity. These findings could suggest the usefulness of miR-146a as a prognostic biomarker for RA.

A previous study was in consistent with our results and reported that high miR-146a expression levels correlated with active disease, whereas low expression levels correlated with inactive disease **(Pauley et al., 2008)**. In addition, **Abou-Zeid et al. (2011)** reported that miR-146a expression levels positively correlated with the indicators of the disease activity and hence, it could be used as a

prognostic biomarker for RA. Furthermore, similar results were reported by **Elsayed et al. (2016)**.

In contrast to our results, a previous study reported that plasma level of miR-146a expression inversely correlated with clinical indices, such as TJC and DAS28 score **(Murata et al., 2010)**. Different types of samples obtained and different timing of samples collection could explain the discrepancy between their results and ours.

In the current study, miR-146a expression levels showed highly statistically significant differences among the different grades of Larsen score, being highest in grade 1 with low radiological damage and lowest in grade 5 with irreversible joint damage. Our findings were in concordance with **Niimoto et al. (2010)**who stated that the expression of miR-146a was high in PBMCs of patients with low score of Larsen grade and patients with a high score of Larsen grade showed a low expression level of miR-146a.

This could be attributed to the negative feedback loop regulation in whichincreased expression of miR-146a leads to down-regulation of IRAK1 and NF-κB,which in turn repressosteoclastogenesis and decrease joint destruction. It is worth to be mentioned that the therapeutic application of miR-146a in RA originates from its inhibitory effect on both IRAK1 and NF-κB**(Saba et al., 2014).**

Our study showed that TLR4 expression was significantly elevated in PBMCs of RA patients, as compared with healthy controls. Consistent with our data, several previous studies confirmed increased expression of TLR4 in RA peripheral blood monocytes as well as in RA synovial tissue and synovial fluid macrophages **(Iwahashi et al., 2004, Radstake et al., 2004, Huang et al., 2007 and Sørensen et al., 2008)**. In addition, **Kim et al. (2007)** reported increased TLR4 in synovial fibroblasts of RA patients compared to those from patients with osteoarthritis. Furthermore, it was found that there was increased responsiveness to the microbial TLR4 ligand LPS in PBMCs from patients with recent onset RA**(Huang and Pope, 2009).**These findings could support the role of TLR4 in RA pathogenesis.

This study revealed that TLR4 expression levels in PBMCs of RA patients showed significant positive correlations with the morning stiffness durations, VAS score, ESR, CRP, TJC, SJC, anti-CCP

antibody and RF titers. Furthermore,our study showed that there was highly statistically significant differences in the peripheral blood TLR4 expressions according to DAS28 being higher in patients with more severe disease activity and lower in those with low disease activity.

These results could be explained by**Radstake et al. (2004)** who stated that TLR4 is a transmembrane receptor that, when triggered by endogenous and/or exogenous ligands, initiates activation of NF-kB leading to production of numerous pro-inflammatory cytokines and chemokines and so contributing significantly to disease activity during disease progression.

In the present study, there was highly statistically significant differences in TLR4 expression levels according to radiological grades of RA patients being higher in grade 5 patients and lower in those with grade 1. These results were reported by **(Roodsaz et al., 2008)** who conducted their study on experimental streptococcal cell wall model and proved that TLR4 was important in the destructive phase, and it contributed to matrix metalloproteinase-mediated cartilage damage and osteoclast formation. Normally, activation of a counter mechanism occurs after every pro-inflammatory response to restore the immunologic balance. However,such a mechanism appears to be insufficient in RA, leading to a vicious circle of activation andcell influx, ending in total joint destruction **(Roelofs et al., 2005)**.

Our results revealed that miR-146aexpression levels negatively correlated with that ofTLR4, a finding consistent with**Yang et al. (2011)**who used a bioinformatics approach and showed a possible interaction between miR-146a and TLR4. In addition, **He et al. (2014)** used a luciferase reporter assay and revealed that miR-146a was able to bind directly to the TLR4 3′UTR target sequence, to downregulate TLR4 expression. Conversely, **Li et al. (2013)** suggested that the action of miR-146a is most effective by affecting downstream signaling intermediates such as IRAK1 and TRAF6 rather than targeting TLR4 mRNA.Anyway, there is a negative feedback loop regulation,involving TLR4 and miR-146a, to tone down the immune response and so miRNAs are considered as ‘fine-tuners’ of the immune response **(Saba et al., 2014).**

In the current study, the accuracy of miR-146a was superior to that of TLR4 in differentiating between different grades of DAS28.MiR-146a

ROC curve showed highest sensitivity and specificity (100%) (AUC: 1.0 at a cut off value of ⩾0.17) in differentiating between high and moderate grades, suggesting its value as a potential biomarker for RA disease activity. Interestingly, to our knowledge, there was no previous comparative study of miR-146a and TLR4 regarding their performance in predicting RA disease activity.

As stated before, DAS28 didn't take into account the status of ankles and feet joints, irreversibly damaged joints and concomitant fibromyalgia. However, the results of the present study showed significantly higher expression levels of miR-146a and TLR4 in PBMCs of RA patients with ankles and/or feet joints involvementthanthose without involvement, a finding that provides an advantage to miR-146a and TLR4 expression estimation over DAS28.The expression levels of TLR4 in patients with concomitant fibromyalgia were higher than those without but did not reach statistical significance.In fibromyalgia, LPS and the released inflammatory mediators activate TLR4 causing its upregulation leading to microglial and astrocytes activation which in turn promotes excitatory glutaminergic neurotransmission causing central sensitization to pain **(Littlejohn, 2015)**.

Based on our findings, it is now clear that miR-146a, and to lesser extentTLR4, can avoid DAS28 limitations and hence, may replace it in clinical practice.However, Validation in larger study groups before clinical application is recommended.

CONCLUSION

Both miR-146a and TLR4 were upregulated in PBMCs of RA patients and were correlated with clinical and laboratory indices as well as DAS28 and Larsen score. However, miR-146a exhibited better performance in predicting RA disease activity, a finding that may nominatemiR-146a to be a promising biomarker for global assessment of RA disease activity thus avoiding subjectivity ofDAS28 and its other limitations.

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الملخص العربى

التعبير الجينى للحامض النووى الريبوزى المتناهى الصغر – ١٤٦ أو مستقبلات شبية التول ٤ فى التنبؤ بنشاط مرض التهاب المفاصل الروماتويدى

رباب فوزى سالم١ ، تهامى حسن ٢ ، بسنت النادى ٣

١ قسم الكيمياء الحيوية الطبية ، كلية الطب ، جامعة بنها
٢ قسم جراحة العظام ، كلية الطب ، جامعة الأزهر
٣ قسم الروماتيزم والتأهيل ، كلية الطب ، جامعة بنها

يعد مؤشر النشاط المرضى ٢٨ (الداس ٢٨) هو الأسلوب الأكثر أستخداماً على نطاق واسع لمرض ألتهاب المفاصل الروماتويدى فى تقييم نشاط المرض ، ومع ذلك فأنة لايعكس نشاط المرض بدقة لانه يعتمد فى التقييم الصحى الذاتى للمريض وتقييم عدد المفاصل المتورمة والمؤلمة عند الضغط عليها ، وبالتالى فأن العثور على دلالات حيوية جينية بالدم مناسبة للرصد الدقيق لنشاط المرض أصبح أساسيا ، لذلك كان الهدف من هذة الدراسة تقييم التعبير الجينى للحامض النووى الريبوزى المتناهى الصغر -١٤٦ أو مستقبلات شبية التول ٤ فى خلايا الدم البيضاء وحيدة النواة لمرضى ألتهاب المفاصل الروماتويدى ودراسة قيمتة كدلالة حيوية جزيئية محتملة لنشاط المرض بالمقارنة مع نتيجة الداس ٢٨ ، وقد أجريت هذة الدراسة على ٥١ مريضا مصابا بالتهاب المفاصل الروماتويدى و١٥ من الاصحاء مناسبين من ناحية العمر والجنس كمجموعة ضابطة . وقد تم تحديد مستويات التعبير الجينى النسبية للحامض النووى الريبوزى المتناهى الصغر -١٤٦ أو مستقبلات شبية التول ٤ بتفاعل البلمرة التسلسل الكمى وكانت هناك أختلافات ذات دلالة أحصائية عالية بين المرضى والأصحاء فيما يتعلق بالتعبير الجينى للحامض النووى الريبوزى المتناهى الصغر -١٤٦ أو مستقبلات شبية التول ٤ . وبالاضافى اللى ذلك لوحظت اختلافات ذات دلالة أحصائية عالية بين المجموعات الفرعية من المرضى فيما يتعلق بالتعبير الجينى للحامض النووى الريبوزى المتناهى الصغر -١٤٦ ومستقبلات شبية التول ٤ . وعلاوة على ذلك أظهر التعبير الجينى للحامض النووى الريبوزى المتناهى الصغر -١٤٦ ومستقبلات شبية التول ٤علاقة ايجابية ذات دلالة أحصائية مع مدة التصلب الصباحى وعدد المفاصل المؤلمه بالضغط عليها وعدد المفاصل المتورمه وقيمة مقياس النظير البصرى ، وكذلك معدل ترسيب خلايا الدم الحمراء والسى بى أر ومضادات السى بى سى والداس ٢٨ . هذا وقد أظهر الحامض النووى الريبوزى المتناهى الصغر -١٤٦ أفضل خصائص فى الأداء وخاصة فى التفريق بين درجات نشاط المرض العالية والمعتدلة . والتى تبين أعلى حساسية وخصوصية وهناك زيادة ذات دلالة أحصائية فى مستوى التعبير للحامض النووى الريبوزى المتناهى الصغر -١٤٦ ومستقبلات شبية التول ٤ فى مرضى التهاب المفاصل الروماتويدى المصحوبين باصابة فى مفصل الكاحلين والقدمين مقارنة مع الذين بدون هذة الأصابة وتخلص هذة الدراسة الى أن التعبير الجينى للحامض النووى الريبوزى المتناهى الصغر -١٤٦ أومستقبلات شبية التول ٤يزيد فى خلايا الدم البيضاء وحيدة النواة بمرضى التهاب المفاصل الروماتويدى وكا مرتبطا مع نشاط المرض .