Egypt. J. Chem. **Vol. 68**, No. 3 pp. 379 - 385 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Bioinformatic Analysis of Differentially Expressed MicroRNAs in Egyptian Patients with Early Onset Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease primarily affecting the diarthrodial joints, causing joint destruction and irreversible joint damage. We aimed to identify the top deregulated miRNAs that might be related to RA pathogenesis followed by bioinformatic analysis to detect their target genes. The study included four RA patients and two healthy controls. Total RNA was isolated from plasma and subjected to miRNAs profiling by next generation sequencing (NGS). Sequencing libraries were constructed and generated using the NEBNextR, UltraTM small RNA Sample Prep Kit for Illumina R (NEB, USA), according to the manufacturer's instructions. Functional annotation of target genes was performed using Enrichr available at https://maayanlab.cloud/Enrichr/, and visualized using Appyter. The top upregulated miRNAs were: hsa-miR-6724-5p, hsa-miR-1469, hsa-miR-4632-5p, hsa-miR-3960, hsa-miR-6815-3p, hsa-miR-6823-3p, hsa-miR-10400-5p, hsa-miR-194-3p, hsa-miR-4301, hsa-miR-6758-5p, their predicted target hub genes were SH3TC2, TM9SF4, HDGF and GPAT4. The top downregulated miRNAs were: hsa-miR-1468-5p, hsa-miR-6510-3p, hsa-miR-6743-5p, hsa-miR-486-3p, hsa-miR-15b-3p, hsa-miR-128-3p, hsa-miR-328-3p, hsa-miR-4493, hsa-miR-4433a-3p, hsa-miR-4433b-5p, their predicted target hub genes were NPTXR, SZRD1, WEE1 and MKNK2. The selected pathways of RA-specific genes were mainly: RA KEGG pathway, IL-17 signaling pathway, Jak-STAT signaling pathway, TH-17 cell differentiation, and MAPK signaling pathway. The predicted target genes of the deregulated miRNAs were found to be involved in RA pathogenesis

Keywords: microRNAs; bioinformatic; Rheumatoid Arthritis; Egyptian; early onset

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic, inflammatory, autoimmune disorder primarily affecting the articular joints, and may be associated with extra articular manifestations. The main hallmarks of RA pathology are synovial hyperplasia, persistent inflammation, articular cartilage degradation and bone erosion [1]. The etiology and pathogenesis of RA are multifactorial, involving interaction between immune system, genetic and environmental factors. Genetic factors contribute to about 40%-60% of the risk and is responsible for persistent and destructive polyarthritis [2].

In the past decade, role of epigenetics in susceptibility to RA received much attention. The most commonly studied epigenetic in RA were the microRNAs (miRNAs) which are single stranded, 20-22 nucleotides, non-coding RNAs. They regulate gene expression at post-transcriptional level. Their main action is negative regulator, repressing the expression of the target genes. Single miRNA can target several genes while each gene can be targeted by several miRNAs [3]. MicroRNAs constitute a large and complex regulatory network playing vital role in several biological processes including cell proliferation, differentiation, apoptosis, regulation of immune system, fat metabolism and hematopoietic differentiation [4]. Several miRNAs are expressed in the cells of innate and adaptive immune systems thereby regulating their development and function including pro-inflammatory cytokine secretion; as a result, they play a role in the induction and suppression of autoimmune diseases [5].

Aberrant miRNA expression has been reported in RA patients and was found to have a role in disease pathogenesis causing joint inflammation, synovial proliferation, destruction and release of inflammatory mediators [6]. Moreover, aberrant miRNAs expression showed association with clinical parameters and can predict therapeutic response [7,8]. Understanding

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Received date 26 May 2024; Revised date 04 July 2024; Accepted date 16 July 2024

DOI: 10.21608/EJCHEM.2024.292925.9773

the molecular pathogenetic mechanisms of RA is necessarily for early diagnosis, and proper treatment through the development of personalized therapy.

Three primary techniques are used to study miRNA expression: polymerase chain reaction (PCR), microarray and sequencing. Next generation sequencing (NGS) technique studies the whole genome sequencing, it is more convenient and easier technology for the discovery of novel circulating miRNAs, moreover it can sequence multiple samples at one time. In addition, NGS is more sensitive and specific than microarray technology and lack the background noise and cross-hybridization [9].

In the present study we aimed to identify deregulated miRNAs in patients with early RA using NGS technique, and their predicted target genes, through bioinformatics approach, in order to search for disease related biomarkers and to develop new drug targets.

2. Patients and Methods

2.1. Patients

The study included four early onset, treatment naïve, RA patients, and two healthy volunteers. Patients were collected from the outpatient rheumatology clinic of the Medical Services Unit of the National Research Centre. All participants were female. Rheumatoid arthritis was diagnosed according to 2010 EULAR criteria [10].

Exclusion criteria were patients with diabetes, infection, major organ disease, malignancy, pregnant female any autoimmune disease or joint diseases other than RA.

Participants signed an informed consent. The study has been approved by the ethical committee of the National Research Centre (n=20152) and has been performed in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

2.2. Laboratory method

Two ml of venous blood samples were collected from all participants in sterile ethylenediaminetetra acetic acid (EDTA)-coated blood collection tubes. To harvest cell-free plasma, blood samples were centrifuged at 4500 ppm for 10 minutes, and the supernatant plasma was removed and stored immediately at -80˚C until analysis**.**

2.2.1.MiRNA extraction

Total short RNA extraction was carried out using miRNeasy kit (Qiagen) according to the manufacturer's protocol designed for serum/plasma samples. Total RNA quantity was evaluated using a Fluorimeter Denovix for quantification before Next Generation Sequencing (NGS). Purified RNA was stored at -80˚C until processed.

2.2.2.MiRNA high throughput sequencing

Library preparation

RNA samples (2.5 ng) were used as input material to prepare libraries for RNA sequencing. Sequencing libraries were constructed and generated using the NEBNextRUltraTM small RNA Sample Prep Kit for Illumina R (NEB, USA), according to the manufacturer's instructions. Index codes were added to attribute sequences to each individual sample. Finally, PCR products were purified using the AM Pure XP system (AMPure XP system); library quality was evaluated by checking the size distribution of the final library using the Bioanalyzer DNA assay. The concentration was assessed using the Qubit fluorometric assay for DNA. After this final step, the library was sequenced using MiSeq flow cell.

Next generation sequencing (NGS)

Quality of samples were checked using fastqc and multiQC. Samples adaptors were trimmed using cut adapt then aligned using bowtie to miRbase database. All counts files were then merged together using a python script. Output counts data were then analyzed using R script hich performed the following; 1. Quantile normalization. 2.Calculating log2 fold change and difference between normalized counts. 3.Plotting top 20 miRNAs according to sorted absolute difference of counts and lollipop plot of top 10 miRNAs according to log2 fold change

2.3.Target gene prediction and functional annotation of the miRNAs

The Mienturnet online tool was used to extract the target genes of the deregulated miRNAs from miRTarBase database. Functional annotation of each gene list was done using Enrichr available at https://maayanlab.cloud/Enrichr/, and visualized using Appyter. The functional enrichment involved Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, molecular functions and biological processes.

2.3.1Selection of hub genes

The most relative pathways for RA were selected from the lists of enriched pathways for the RA-specific genes and the hub genes were picked up.

Results

Four early onset RA patients, treatment naïve, and two healthy volunteers were included in the study. All participants were female, the age of the patients ranged from 37 to 51 years, mean 42.5 ± 5.6 years. The age of the controls ranged from 39 to 51 years, mean 45±6 years. Duration of the disease ranged from 4 to 8 months. Disease activity score-18 (DAS-28) ranged from 5.9 to 6.0, mean 5.9 ±0.06.

3.1.Top deregulated miRNAs

The top 10 up-regulated and 10 down-regulated miRNAs in RA patients compared to controls are presented graphically as Lolliplot (figure 1). The heatmap showing the results of the functional annotation of the top deregulated miRNAs is shown in figure 2 for the upregulated miRNAs and figure 3 for the downregulated miRNAs.

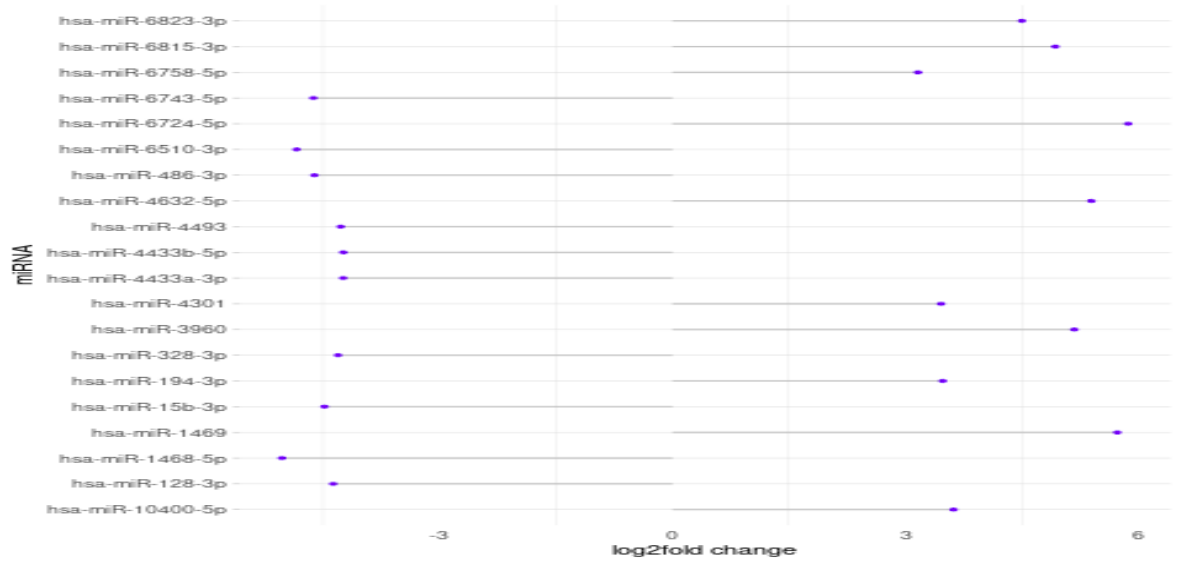

Figure 1: Lolliplote represents the top 10 up and down differentially expressed miRNAs in RA patients compared to controls based on log2fold change and P value

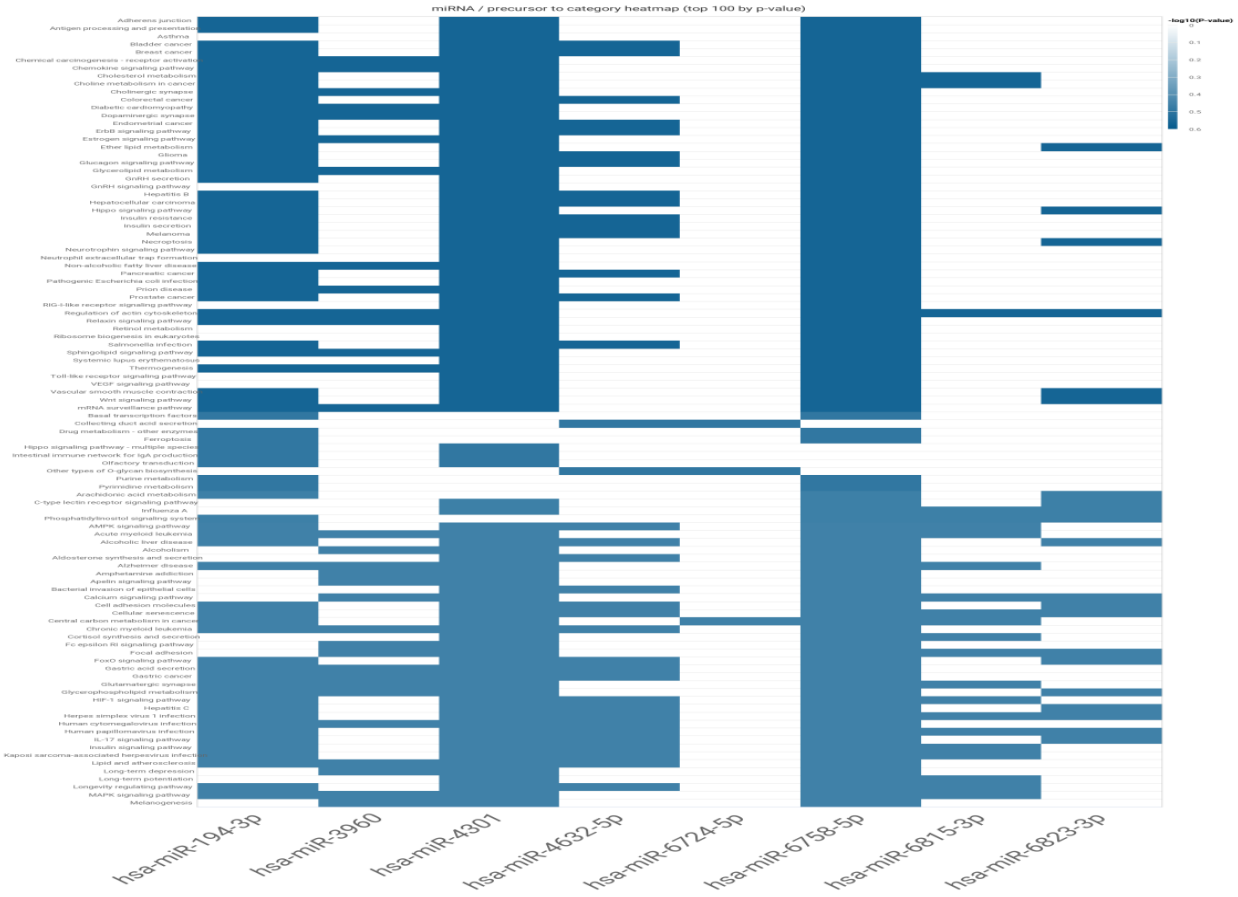

Figure 2 : Heatmap of upregulated miRNAs showing their enriched pathways and gene ontology(GO) terms drawn by the miRNA enrichment analysis and annotation tool (miEAA 2.0)

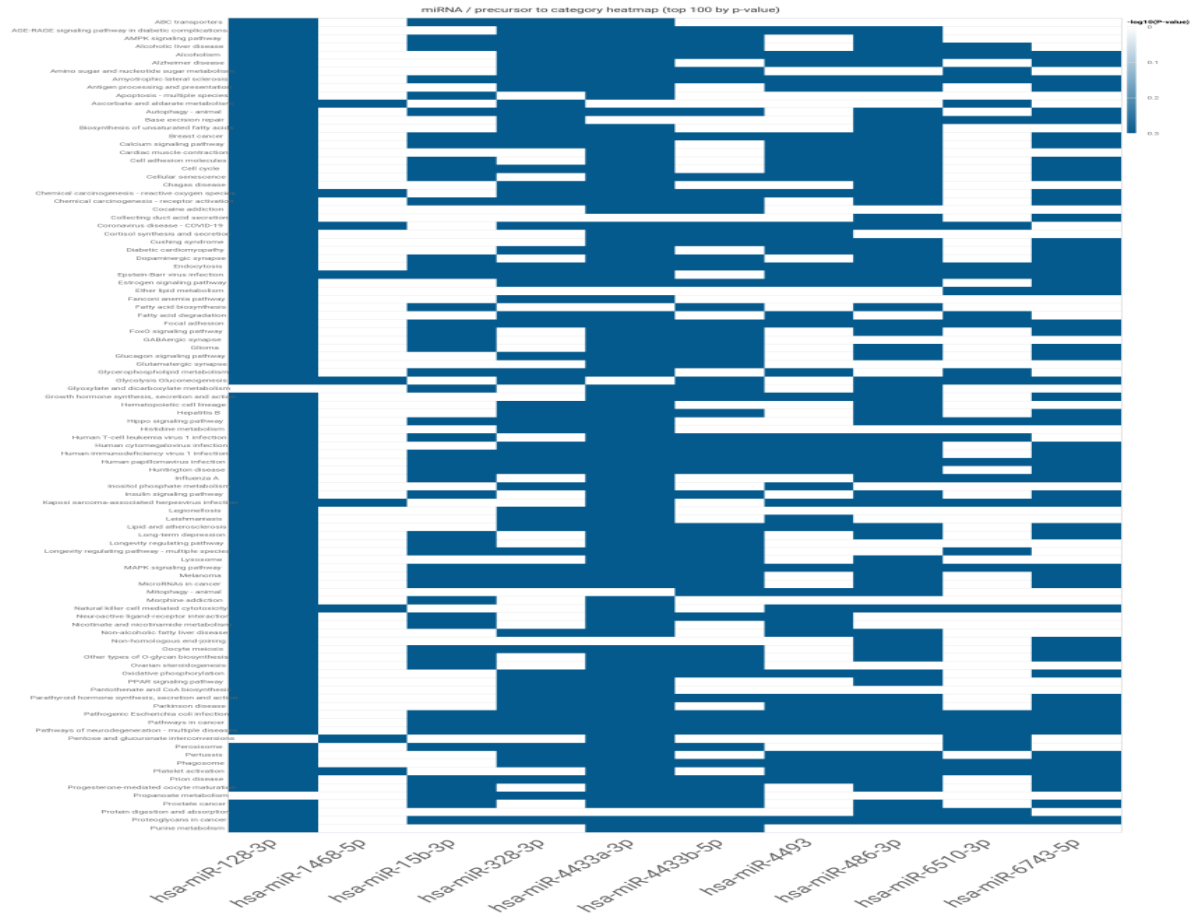

Figure3: Heatmap of dowregulated miRNAs showing their enriched pathways and gene ontology (GO) terms drawn by the miRNA enrichment analysis and annotation tool (miEAA 2.0)

3.2. Target gene prediction

Functional annotation using Mienturnet by miRTarBase was done. The target genes of the deregulated miRNAs (DEMs) in RA patients compared to controls were extracted based on the quantity of interactions (≥2). It showed that the 10 up-regulated miRNAs were predicted to target 57 genes and the most relevant genes were SH3TC2, TM9SF4, HDGF and GPAT4. On the other hand, 168 genes were predicted as target genes of the 10 down-regulated miRNA and the most relevant genes were NPTXR, SZRD1, WEE1 and MKNK2.

3.3. Functional pathways analysis in Kyoto Encyclopedia of Genes and Genomes (KEGG)

The highly enriched pathways of the target genes of the deregulated miRNAs, in KEGG database are: insulin signaling pathway, neurotrophinsignaling pathway, glucagon signaling pathway and central carbon metabolism in cancer.

3.4. Gene ontology (GO): Molecular Functions (MFs)

Regarding the gene ontology: molecular functions, the highly enriched MFs are transcription and nucleic acid binding.

3.5. Gene ontology: Biological Processes (BP)

Regarding the gene ontology: biological processes, the highly enriched BP is regulation of transcription.

3.6. Selection of hub genes related to RA pathogenesis

Ten pathways, related to RA pathogenesis, were selected from the list of enriched pathways of target genes. The selected pathways were mainly: RA KEGG pathway, IL-17 signaling pathway, Jak-STAT signaling pathway, TH-17 cell differentiation, and MAPK signaling pathway .

The genes involved in each pathway that are predicted tobe targeted by the deregulated miRNAs in RA were extracted and the repeated genes were deleted. As a result, 15 hub genes were selected and their predicted target miRNAs are listed in table 1.

Table 1: The selected hub genesrelated to RA pathogenesis and the deregulated miRNAs targeting them.

Gene symbol	miRNAs	regulation
CEBPB	miR-486-3p, miR-6743-5p	down
YWHAE	miR-6758-5p,miR-194-3p	up
SLC7A5	miR-194-3p, miR-6724-5p	up
PIAS4	miR-3960, miR-6815-3p	up
SP90AB1	miR-6758-5p, miR-194-3p	up
TBL1XR1	miR-4433b-5p, miR-4433a-3p	down
MKNK2	miR-128-3p, miR-486-3p, miR-6743-5p, miR-4433b-5p	down
TP6V1E1	miR-486-3p, miR-6743-5p	down
SYK	miR-1468-5p, miR-486-3p	down
HSP90B1	miR-128-3p, miR-4433a-3p	down
RXRA	miR-128-3p, miR-486-3p	down
RAF1	miR-486-3p, miR-6743-5p	down
SOCS7	miR-4632-5p, miR-6815-3p	up
CAMK2A	miR-486-3p, miR-6743-5p	down
APK8IP3	miR-3960, miR-194-3p	up

4. Discussion

MicroRNAs have been identified as important regulators of immune system function and have been reported to be deregulated in several autoimmune diseases and cancer. In this study, we used NGS to identify the top differentially expressed miRNAs that were up/down-regulated in RA patients compared to healthy individuals, then, through bioinformatics, predicted target genes and signaling pathways for RA were reviewed. In addition, through GO and KEGG pathway analyses, we selected the hub genes from each list by selecting pathways that were identified as contributors to RA pathogenesis. To the best of our knowledge, the selected miRNAs were not previously reported deregulated in RA.

After reviewing the literature, 10 pathways were selected from the list of enriched pathways of target genes **[11].**
The downregulated miR-486-3p and miR-6743-5p are predicted to target six of those genes which are: SYK, CaMKII, CEBPB, MKNK2, RAF1 and ATP6V1E1. Spleen tyrosine kinase (Syk) is a member of the Src family of non-receptor tyrosine kinases, and it is an important molecule for regulation of several signal transduction pathways that are involved in the pathogenesis of autoimmune diseases especially autoantibody-mediated such as RA. It is highly expressed in hematopoietic system. Activation of SYK affects several biological processes such as cytokine production, antibody-coated cells phagocytosis and maturation of osteoclasts. In RA patients, SYK levels in peripheral blood B cells were higher than controls, moreover their levels were strongly related to anti-citrullinated protein antibodies **[12].**

In the same context, CaMKII (calcium/calmodulin-dependent protein kinases II) is activated in inflammatory diseases such as RA. It is expressed in T cells, macrophages and fibroblasts of RA synovial tissue **[13].**CaMKII promotes bone resorption through its effect on osteoclastic differentiation. It induces inflammation through enhancing proinflammatory cytokine production by macrophages **[14,15].**
C/EBPβ (CCAAT/enhancer-binding protein β) is a transcription factor that is activated in RA synovium and promotes expression of various matrix metalloproteinases. C/EBPβ plays a pivotal role in bone destruction in RA as it stimulates osteoclastogenesis and promotes production of pro-inflammatory cytokines **[16,17].**

In RA, RAF1 (Serine/Threonine Kinase) is associated with increased adhesion molecules. RAF1 is regulated by the phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathway which is involved in the pathogenesis of RA. Downregulated RAF1 expression inhibited ERK1/2 phosphorylation pathway and inhibited the development of RA. **[18,19]**.
MKNK2 is among the differentially expressed genes (DEGs) in RA **[20].**It is a predicted target gene of four of the downregulated miRNAs in the present study which are miR-128-3p, miR-486-3p, miR-6743-5p and miR-4433b-3p.
Among the down regulated miRNAs in the present study weremiR-486-3p, and miR-6743-5p which target *ATP6V1E1* (ATPase H+ transporting V1 subunit E1) gene. *ATP6V1E1* is associated with osteoclastic activity, that causes bone resorption, and play a role in inflammatory responses such as cytokine secretion, phagocytosis and neutrophil granule exocytosis **[21].**

The *HSP90B1* gene is a predicted target gene of miR-128-3p and miR-4433a-3p which were downregulated in the present study among RA plasma. *HSP90B1* gene encodes glucose-regulated protein 94/endoplasmin (HSP90B1) which is one of the HSP90 family members and plays important role in autoimmune disease through different mechanisms **[22]**. Under physiologic conditions, GRP94/endoplasmin improves B cells function by chaperoning TLRs **[22]**. In case of diseases, they are transferred to the surface of the cell and extracellular space and act as autoantigen inducing antibodies formation and hence immune system activation. In addition, they promote chronic inflammation through their action as endogenous ligand of toll-like receptor2(TLR2). *HSP90B1* is over-expressed in RA synovium and isrelated to synovial inflammation. **[23,24,25].**

Retinoid X receptor alpha (RXRA) and MAPK8IP3 were found significantly differentially methylated in RA**[26]**. RXRA is important regulator of innate immunity and is considered as potential target for immunotherapy in RA **[27].** RXRA is a predicted target gene of miR-128-3p and miR-486-3p which were downregulated in our results in RA plasma.
miR-6758-5p and miR-194-3p, which were upregulated in our results in RA plasma, are predicted target for YWHAH gene. the YWHAH gene encodes for 14-3-3 eta protein. YWHAH 14-3-3 η plays a role in RA pathogenesis as it is upregulated in RA synovial fluid and tissue and contributes to joint destruction in RA patients
[28, 29].A recent study on Egyptian RA patients, genetic variants of YWHAH were associated with increased hypoxia and angiogenesis and with disease severity **[30]**.

SLC7A5 (solute carrier family 7 member 5) is an amino acid transporter. Activated NF-κBsignaling pathway causes over expression of SLC7A5 in synovial tissue of RA patients compared to osteoarthritis patients. The upregulated SLC7A5 promotes over expression of matrix metalloproteinase-1 (MMP-3) and MMP-13 in RA fibroblast-like synoviocytes indicating potential role of SLC7A5 in RA pathogenesis **[31].**

Plasma miR-4632-5p and miR-6815-3p were upregulated in the present study and they are predicted target for suppressor of cytokine signaling (SOCS) gene. The SOCS proteins are intracellular proteins consisting of eight members: SOCS1 up to SOCS7 besides the CIS protein and their expression is promoted by cytokines through activated STATs (32). Isomäki and colleagues **[32],** reported over- expression of SOCS1 mRNA in synovial tissues from RA patients compared with osteoarthritis patients. The SOCS family has an important immune and inflammatory regulatory effect and is responsible for the development and progression of arthritis. In chronic RA over expression of SOCS may significantly alter cellular responsiveness to cytokines, and may participate in disease progression by causing unresponsiveness to anti-inflammatory cytokines **[33].**

HSP90AB1was identified as anRA-associated genes by gene-based association study and is a predicted target gene of miR-6758-5p and miR-194-3p, which were upregulated in our studied RA patients. The *HSP90B1* gene encodes glucose-regulated protein 94 (GRP94) a member of the HSP90 family. GRP94/endoplasmin has significant role in inflammatory process and contributes to autoimmune diseases. In disease state, GRP94 translocates to the surface of the cell and the extracellular space and acts as autoantigen inducing antibodies formation. Moreover, it is overexpressed in RA synovium and correlates with inflammatory score **[22,24].**

In the present study, miR-3960 and miR-6815-3p were upregulated. They are predicted to target Protein Inhibitor of Activated STAT (PIAS), whose family members include PIAS1, PIAS2 (PIASx), PIAS3, and PIAS4 (PIASy). PIAS has regulatory roles in various cellular events, including cell survival, cell migration, and signal transduction in several cell types. Lao and colleagues, observed increased expression of PIAS3, but not PIAS1, PIAS2, or PIAS4, in RA fibroblast-like synoviocytes (FLSs) and in synovial tissues suggesting its contributing role in cartilage destruction in RA [**34**] .

The top deregulated miRNAs in early onset RA, identified through NGS, were found to target genes related to RA pathogenesis through regulating biological processes, and signal pathways. Identification of deregulated miRNAs in RA provides a way for early diagnosis and proper management of RA and provides opportunities for precision medicine.

5. Conflicts of interest: None of the authors declared Financial and Non-Financial Relationships and Activities, and Conflicts of Interest regarding this manuscript. The sponsor had no role in study design, data collection, data analysis, data interpretation or writing of the report.

6. Funding: This study was funded by the Science, Technology and innovation Funding Authority, Egypt, Grant No. 26293.

7. Acknowledgments: The authors thank the Science, Technology and innovation Funding Authority for funding the study

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