

Downregulation of Drosha, Dicer, and DGCR8 mRNAs in Peripheral Blood Mononuclear Cells of Patients with Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a systemic autoimmune disorder causing irreversible joint damage. MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that degrade or translate inhibition of mRNAs. miRNAs can be used as therapeutic targets and predictive biomarkers in many disorders. This study was undertaken to investigate whether or not the expression of key elements in miRNA biogenesis, Drosha, DGCR8 and Dicer mRNAs is dysregulated in RA patients.

In this case-control study, 50 patients with RA and 50 age- and gender-matched healthy subjects participated. The peripheral blood mononuclear cells (PBMCs) were separated from the whole blood, the total RNA content of the cells was isolated and the first strand cDNA was synthesized. Quantitative analysis was performed through real-time polymerase chain reaction (PCR) using SYBR Green gene expression master mix to detect mRNA level expression of Drosha, DGCR8 and Dicer.

The expression levels of Drosha and DGCR8 were significantly downregulated in patients with RA in comparison with the healthy controls (P value = 0.043, P value = 0.000365, respectively). The expression level of Dicer was downregulated in RA patients when compared to the healthy controls, although the difference in expression was not significant (P value= 0.156). RA patients with a familial history of autoimmune rheumatic disease recorded significant overexpression of all three genes. Moreover, DAS28 was significantly correlated with mRNA expression of Drosha, Dicer and DGCR8.

The data suggests that downregulated expression of Drosha, DGCR8 and Dicer mRNAs may be contributing to the pathogenesis of RA.

Keywords: Rheumatoid arthritis; micro RNA; gene expression; Drosha; Dicer; DGCR8

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disorder characterized by progressive joint inflammation which affects tissues and organs. It is estimated that 1% of the world population is affected by RA and its prevalence is higher in females than in males [1]. This progressive disease terminates in systemic complications, disability, high medical and social costs, high morbidity and mortality rates and influences the quality of life [2]. The precise etiology of RA is not fully understood; however, it is thought that the interaction of genetic, immunological and environmental factors (such as smoking, hormones, infection and microbiota) contributes to development of the disease [3]. Genetic variants are thought to be responsible for 50% to 60% of the risk of developing RA [4]. Concordance rates among monozygotic and dizy-

gotic twins for RA were estimated to be 12% to 15% and 3.5%, respectively, suggesting the importance of genetics in the etiopathogenesis of the disease.

Epigenetics, the constant and inherited changes in gene expression without alteration in DNA sequences [5] may also play a role in RA. Epigenetic processes include DNA methylation, histone modifications and microRNA (miRNA). miRNA is the single-stranded, highly conserved, noncoding and endogenous RNA of 20-22 nucleotides in length that bind to the 3'-untranslated region (3'-UTR) of their determined target genes and promote either translational prevention or mRNA degradation [6,7]. miRNAs have emerged as important regulators of the immune response, cell differentiation, cell cycle, apoptosis and immune functions [8-10]. They are believed to have crucial roles in the pathogenesis of human inflammatory arthritis

such as RA [11,16].

In the nucleus, genes encoding miRNAs are transcribed to a long primary transcript (pri-miRNAs; several hundred nucleotides) by the action of RNA polymerase II/III. Pri-miRNA comprises a hairpin stem, terminal loop and 5' and 3' single-stranded RNA extensions [17]. Pri-miRNA undergoes two-step sequential processing. First, it is cropped into hairpin-shaped pre-miRNAs (60-70 nt stem-loop structure) by the Drosha/DiGeorge syndrome critical region 8 (DGCR8 or Pasha) complex, the nuclear RNase III enzyme. The catalytic subunit of the microprocessor complex, a combination of Drosha and DGCR8 [18], is Drosha and DGCR8 stabilizes Drosha by a protein-protein interface and identifies the RNA substrate [19]. Transportation of the pre-miRNA from the nucleus to the cytoplasm is performed by the exportin-5 (Exp5) through the nuclear pore complex [12].

Next, the hairpin-shaped pre-miRNA is cut by cytoplasmic RNase III Dicer/TAR-RNA binding protein (TRBP) complex, which removes the hairpin loop and generates ~22 nt of unstable mature miRNA duplex [20, 21]. A single strand of the miRNA duplex is incorporated into a 200-500 kD of ribonucleoprotein effector complex known as the RNA-induced silencing complex (RISC). This complex has endonuclease activity and is capable of modulating gene expression. [22].

The dysregulated expression profiles of some miRNAs display downregulation of miR-124a in RA synovium tissue, upregulation of miR-146a, miR-155, miR-203 in RA synovium tissue, miR-16, miR-132, miR-146, miR-155 in RA peripheral blood mononuclear cells (PBMCs) and overexpression of miR-223 in RA CD4⁺ naive T cells [12]. miRNAs appear to be promising biomarkers in a large panel of diseases, such as RA [23], which is why many studies have concentrated on the involvement of miRNAs on RA development. As the intracellular role of miRNAs is closely related to the function of Drosha, DGCR8 and Dicer, the present study investigated whether or not the expression level of Drosha and Dicer (as main miRNA machinery enzymes) and DGCR8 (as a microprocessor complex component) in PBMCs is involved in the pathogenesis of RA. The results may help achieve a better understanding of the disease pathogenesis and contribute to the development of novel therapeutic strategies for RA.

Materials and Methods

Study design

The RA patients were recruited from the Rheumatology

Research Center of Shariati Hospital in Tehran, Iran. All patients were diagnosed as having RA according to American College of Rheumatology criteria [24]. Fifty RA patients and 50 age- and gender-matched healthy controls without RA or other autoimmune diseases were enrolled in the study. The control group had a normal range of erythrocyte sedimentation rate (ESR). A standard questionnaire was used to collect information regarding age, age at RA diagnosis, symptoms and drug treatment. The Human Research Ethics Committees of Tehran University of Medical Sciences reviewed and approved this study. Informed consent forms were obtained from all subjects.

Sample collection, PBMCs isolation and RNA extraction protocols

About 5 ml of peripheral blood was collected from all participants under sterile conditions using venipuncture and was stored in EDTA anticoagulant. PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation according to manufacture instructions. RNA extraction was carried out using a High Pure RNA isolation kit (Roche; Germany) according to manufacturer instructions. The purity and integrity of the extracted RNA was evaluated using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific; USA) at 260/280 nm absorbance.

Complementary DNA (cDNA) synthesis protocol

Following RNA extraction from PBMCs, first strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche; Germany) according to manufacturer instructions. Briefly, 1 µg of isolated RNA was reverse transcribed in a final volume of 20 µl solution containing 2 µl of primer (random hexamers) and RNase free water. Then the mixture incubated at 65°C for 10 min and cooled on ice. Next, 0.5 µl of RNase inhibitor, 4 µl of reaction buffer, 2 µl of dNTP and 0.5 µl of reverse transcription enzyme was added to the mixture. The reverse transcription conditions were as follows: 25°C for 10 min, 50°C for 60 min and 85°C for 5 min. The cDNA samples were stored at -20°C for later analysis.

Real-time PCR primer design

In this study, the primers were adapted from previously published studies by Sand et al. where mRNA expression of Drosha, Dicer and DGCR8 was measured in epithelial skin cancer subjects [17, 25]. In order to check the accuracy and specificity of each primer, (Drosha, Dicer, DGCR8 and RPL38 as a housekeeping gene) the Basic Local Alignment Search Tool on the NCBI website was used [26]. Table 1 lists the details of each primer.

Table 1. Primers used for real-time gene expression of Drosha, Dicer, and DGCR8

Target	Sequence	Amplicon size (bp)	Temp (°C)
Drosha F	5'-CATGTCACAGAATGTCGTTCCA-3'	115	58.4
Drosha R	5'-GGGTGAAGCAGCCTCAGATT-3'	115	59.8
Dicer F	5'-TTAACCTTTTGGTGTGTTGATGAGTGT-3'	94	58.5
Dicer R	5'-GGACATGATGGACAA TTTTCACA-3'	94	57.1
DGCR8 F	5'-GCAAGATGCACCCACAAAGA-3'	93	57.3
DGCR8 R	5'-TTGAGGACACGCTGCATGTAC-3'	93	59.8
RPL38 F	5'-TCACTGACAAAGAGAAGGCAGAGA-3'	88	61
RPL38 R	5'-TCAGTGTGTCTGGTTCATTCAGTT-3'	88	59.7

Quantitative real-time PCR

Quantitative analysis was performed by quantitative PCR using the SYBR Green gene expression master mix (Takara Bio; Japan) and Step One Plus real-time PCR (Applied Biosystems; USA). The reaction mixture contained a total volume of 20 μ l, including 10 μ l SYBR Green PCR master mix, 2 μ l primers (1 μ l each), 6 μ l RNase free water and 2 μ l cDNA template. The conditions of mRNA Quantitative real-time PCR were as follows: 95°C for 30 sec, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Evaluation of mRNA expression was performed using the comparative CT method previously explained by Livak and Schmittgen [27]. The relative quantification levels of the target mRNAs were normalized versus the RPL38 mRNA transcript level. Ultimately, the relative mRNA expression level for each sample was measured as: relative mRNA expression = $(2^{-\Delta C_t}) \times 1000$.

Statistical analysis

SPSS software version 23 (SPSS; USA) was used to analyze the data. To assess normality, the Kolmogorov–Smirnov test was applied. Independent sample t-test or the Mann-Whitney U test was used to compare groups. For plotting, GraphPad Prism version 7.00 (GraphPad; USA; www.graphpad.com) was used. Scale data were expressed as mean \pm standard error of mean (SEM). Statistical significance was set at P value $<$ 0.05.

Results

The study group comprised 50 RA individuals (9 men with a mean age of 54.66 ± 12.58 years and 41 women with a mean age of 45.85 ± 8.88 years) and 50 unrelated healthy controls. Table 2 provides the demographic specifications of the RA patients.

The expression level of Drosha was lower in the PBMCs of the RA patients (0.78 times downregulated) in comparison with the control group (P value = 0.043; Figure 1A). The DGCR8 expression level in PBMCs from RA patients was also 0.76 times downregulated compared to the controls (P value = 0.000365; Figure 1C). The Dicer expression level in PBMCs of RA patients decreased (0.54 times downregulated) compared to that of the control group (Figure 1B), although the expression difference was not statistically significant (P value = 0.156).

The mRNA expressions of Drosha, Dicer, and DGCR8 were evaluated in the RA patients with respect to differences in clinicopathological and demographic specifications. Male versus female, smoker versus non-smoker, anti-cyclic citrullinated peptide (anti-CCP) positive versus anti-CCP negative RA patients did not show significant differences in the expression of these three genes. However, the rheumatoid factor (RF) positive RA patients demonstrated significant overexpression of Drosha mRNA in PBMCs in comparison with RF negative RA patients (fold change = 1.81; P value = 0.03).

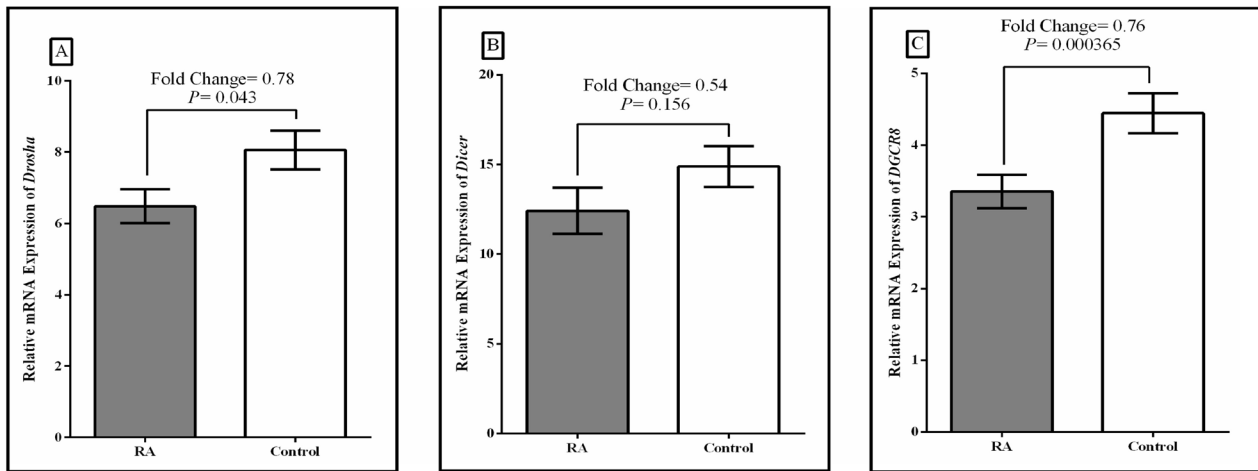


Figure 1. Comparison of relative expression in PBMCs of RA patients and healthy subjects of: (A) Drosha; (B) Dicer; (C) DGCR8.

There was also significant overexpression of Drosha (fold change = 1.89; *P* value = 0.003), Dicer (fold change = 1.66, *P* value = 0.049) and DGCR8 (fold change = 1.40, *P* value = 0.035) mRNAs in RA patients with a familial history of autoimmune rheumatic diseases in comparison with those patients without such a history (Figure 2, Table 3).

In correlation analysis (Figure 3), it was seen that age and ESR of RA patients did not correlate with the mRNA expression level of the three genes. However, the disease activity score for 28 joints (DAS28) correlated significantly with positive trends for mRNA expression levels of Drosha (*r* = 0.56, *P* value < 0.0001), Dicer (*r* = 0.47, *P* value = 0.0001) and DGCR8 (*r* = 0.31, *P* value = 0.027).

Table 2. Demographic data of RA patients

Characteristic	Value
Gender	Female (N=41), Male (N=9)
Age ± SD	47.5 ± 10.1
Age of diagnosis	35.6 ± 10.5
DAS28	3.5 ± 2.0
ESR	35.7 ± 36.2
Smoking	5 (10.2%)
Familial history (autoimmune rheumatic disease)	13 (27.1%)
RF positive	11 (37.9%)
Anti-CCP positive	7 (25.9%)
Prednisolon	17 (34 %)
HCQ	17 (34%)
SSZ	9 (18%)
MTX	29 (58%)

SD, standard deviation; DAS28, disease activity score for 28 Joints; ESR, Erythrocyte sedimentation rate; RF, Rheumatoid factor; CCP, cyclic citrullinated peptide; HCQ, Hydroxychloroquine; SSZ, Sulfasalazine; MTX, Methotrexate.

Table 3. Differences in fold change and *P* values of the expression levels of Drosha, Dicer, and DGCR8 based on different category of patients for clinical specifications

Groups	Drosha		Dicer		DGCR8	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
Male vs. Female	1.01	0.96	0.89	0.59	1.04	0.88
Smoker vs. Non-smoker	0.81	0.43	0.95	0.96	0.95	0.83
With familial history vs. without familial history	1.89	0.003	1.66	0.049	1.40	0.035
RF positive vs. RF negative	1.81	0.03	1.73	0.11	1.40	0.10
Anti-CCP positive vs. Anti-CCP negative	1.53	0.59	2.12	0.41	1.22	0.99

RF, Rheumatoid factor; Anti-CCP, anti-cyclic citrullinated peptide

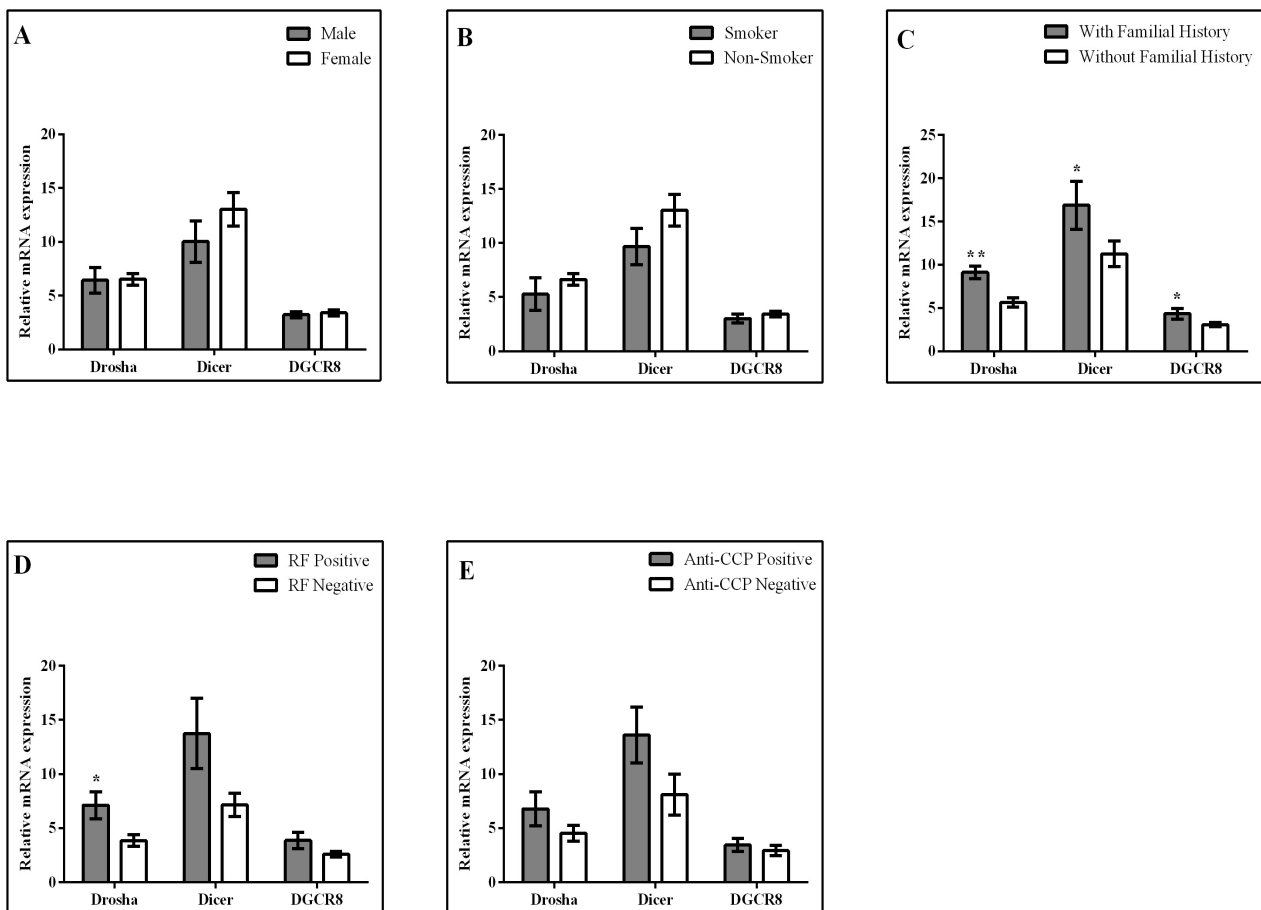


Figure 2. mRNA expression levels of Drosha, Dicer and DGCR8 in RA patients according to clinicopathological specifications of: (A) sex; (B) smoking; (C) familial history; (D), RF; (E) anti-CCP (* and ** implies to *P* < 0.05, and *P* < 0.01, respectively).

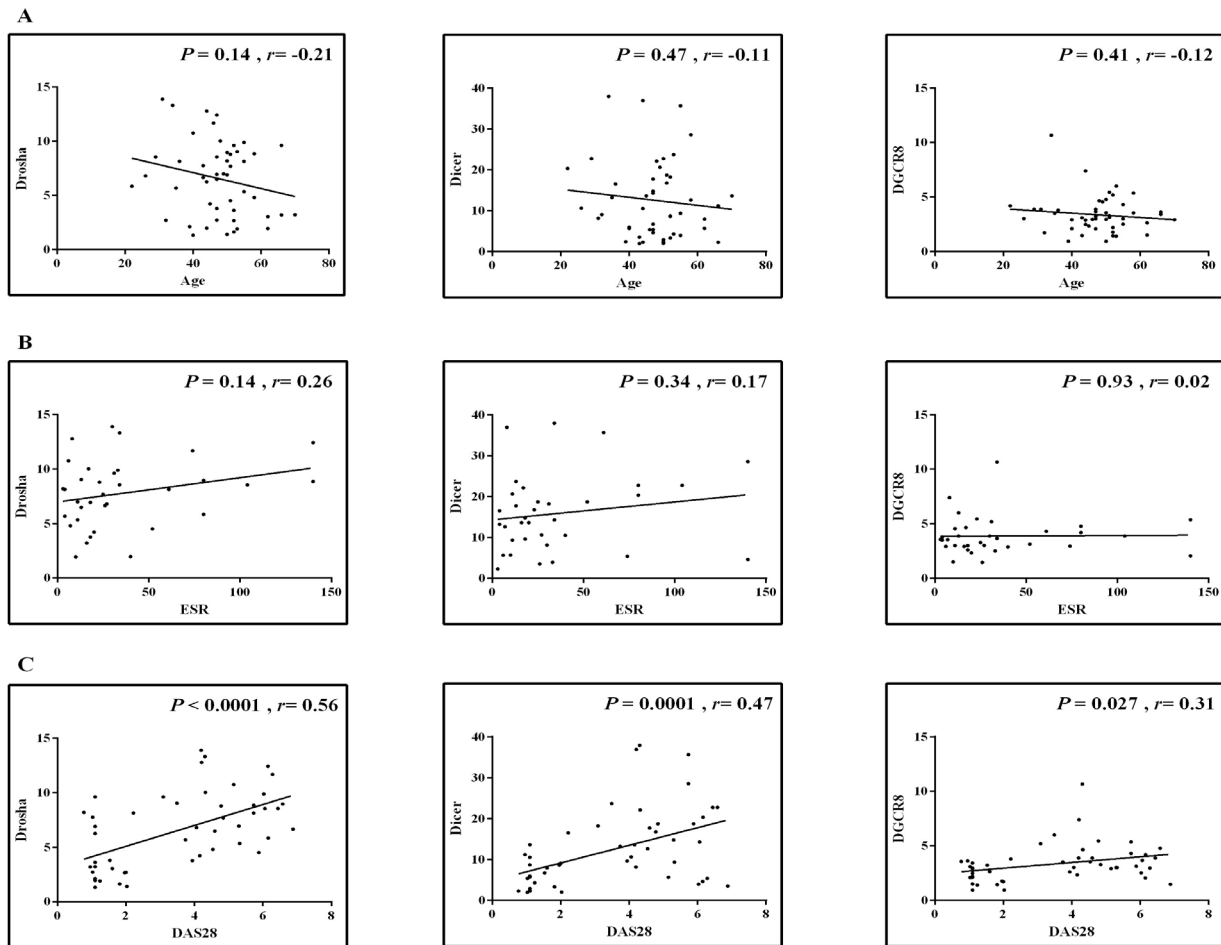


Figure 3. Correlation of mRNA expression levels of Droscha, Dicer, and DGCR8 with age (lane A), ESR (lane B) and DAS28 (lane C).

Discussion

Because previous reports have demonstrated that aberrant expression of miRNA in RA patients, we hypothesize that the elements involved in the synthesis of these molecules might also be dysregulated. To check this hypothesis, we examined the expression levels of Droscha, DGCR8 and Dicer in PBMC samples obtained from RA patients in comparison with healthy controls.

Epigenetics has provided a new orientation linking genetics and environmental factors. miRNA, as an epigenetic marker, has benefits for early stage detection of disease, prediction of disease progression and administration of appropriate treatment approaches; thus, it could serve as diagnostic and prognostic marker for many disorders [28]. miRNA functions as a post-translational regulator of gene expression and is known to be evolutionarily conserved between species [5].

It has been reported that miRNA plays an important role

in many autoimmune diseases, including systemic lupus erythematosus [29], multiple sclerosis (MS) [30], psoriasis [31] and sjögren's syndrome [32], suggesting a potential therapeutic target of miRNA in this group of disorders. Studies have documented dysregulation of various miRNAs in PBMCs [33, 34], serum [35], synovial tissue [36] and macrophages [37] in RA patients. For instance, the expression level of miR-146a, miR-155, miR-132 and miR-16 increases in the PBMCs of RA patients in comparison with healthy control individuals [38].

miRNA machinery is a well-organized process which defines the biogenesis of miRNA [39]. In the pathway of miRNA biogenesis, molecules including Droscha, Dicer and DGCR8 have pivotal roles. Additionally, both Droscha and Dicer have critical roles in the function, differentiation and proliferation of B cells and regulatory T (Treg) cells and, therefore, play roles in immune regulation and pathogenesis of inflammatory disease. Loss of either Droscha or Dicer results in early onset lymphoproliferative disease

and defective FoxP3 induction in Treg cells which leads to autoimmunity [40].

Results of a study have revealed downregulation of Drosha and Dicer in patients with Graves' disease and Hashimoto's disease, suggesting that low expression of these enzymes may cause reduced expression of miRNAs like miR-27b, miR-let7f, miR-21 and miR-98, which will increase the susceptibility to autoimmune thyroid disease [41]. Another study demonstrated significantly upregulation of Drosha, Dicer and DGCR8 in psoriasis patients in comparison with controls [42]. A study performed by Jafari *et al.* revealed significant upregulated expression of Drosha, Dicer and DGCR8 in MS patients in comparison with normal subjects and concluded that overexpression of these elements may contribute to the pathogenesis of MS [43]. These molecules were also dysregulated in patients with ankylosing spondylitis [44].

Our results show significant downregulation of mRNA expression levels of Drosha and DGCR8 in PBMCs of RA patients and the expression level of Dicer was slightly downregulated. These findings suggest that dysregulation of these major miRNA machinery elements may provide new insights for the possible contribution of these factors in RA pathogenesis. In contrast to our results, the study of Wang *et al.* found upregulated mRNA expression levels for Dicer and Drosha in RA patients. They concluded that the activation of Dicer led to suppression and balance of the production of tumor necrosis factor (TNF)- α and suggested that Dicer in RA patients functions as a regulator of immune responses [45]. The discrepancies observed between these two studies may stem from differences in sample sizes, ethnic backgrounds, inclusion and exclusion criteria, methodological approaches, statistical tests and even the effect of drugs and medications.

Array analysis demonstrated that, among the evaluated miRNAs, 91% were overexpressed while 9% were downregulated after anti-TNF α /disease-modifying antirheumatic drug (DMARD) combined therapy. Among the overexpressed miRNAs were miR-16-5p, miR-23-3p, miR125b-5p, miR-126-3p, miRN-146a-5p and miR-223-3p, which were only observed in responder patients [46]. This suggests that therapies in RA patients affect the level of miRNA and may regulate the components of miRNA biogenesis. Nonetheless, we were unable to assess the role of medications in modulating the major components of miRNA biogenesis because all of the patients had received

at least one medication before being included in the study.

Among the clinical characteristics of RA patients, familial history and RF status affected the mRNA expression of Drosha in PBMCs. The RF positive cases showed upregulated expression of Drosha. Moreover, the DAS28 level correlated with Drosha, Dicer and DGCR8 mRNA levels. To prematurely conclude from these observations that dysregulation of miRNA in RA patients (because of the altered level of enzymes in miRNA biogenesis like Drosha) may affect the disease activity (DAS28) and inflammatory status (RF) in patients.

There are some limitations and caveats with respect to the current study. It was conducted only on the PBMCs of RA patients and involved a limited number of patients; thus, it would be proper to conduct other studies with a larger number of participants using other types of samples, such as serum, synovial tissue and macrophages. It should also be noted that disease duration effects the epigenome of patients, especially those taking medication. Moreover, this was a cross-sectional study. A prospective study would be helpful in determining the possible role of environmental factors for the epigenome of patients by modulating miRNA levels, possibly through regulation of miRNA biogenesis components.

Overall, our data reveals that downregulation of key components of miRNA biogenesis, including Drosha, Dicer and DGCR8 is associated with disease activity level and inflammation status. Hence, these components may be involved in the pathogenesis of RA. There is a consensus that miRNAs modulate the inflammatory reactions in the joints and are biomarkers for inflammatory diseases and development of novel disease-modifying therapies. Hopefully, further studies can provide promising research orientations leading to novel insights into immunopathological mechanisms and development of new therapeutic approaches for RA patients.

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Conflict of interest

The authors declare no conflict of interest.

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