VOLUME-8, ISSUE-3, MARCH-2019 • PRINT ISSN No 2277 - 8160

Original Research Paper

Orthopaedics

EARLY BIOMARKER FOR THE DETECTION OF RHEUMATOID ARTHRITIS USING MIRNA EXPRESSION PROFILING IN WHOLE BLOOD - A CASE CONTROL STUDY

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ABSTRACT

INTRODUCTION: Currently the diagnosis for rheumatoid arthritis is based on the 'The American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) criteria 2010, which ultimately aims at early detection of RA. However, this criterion only gets fulfilled when nearly more than ten joints are affected, thus giving rise to false negative results in the earlier phases. Early detection and early intervention tends to have better prognosis compared to delayed diagnosis. Thus a new category of biomarkers which enables early detection is essential. miRNAs are a group of approximately 20 to 22 nucleotides noncoding RNAs, which have been proved to cause regulation of gene expression at the post- transcriptional level. Moreover, when miRNAs are abnormally expressed they can lead to pathological conditions involving the immune system, such as cancer and autoimmunity hence their role as an early biomarker in the diagnosis of RA is evaluated.

METHODOLOGY: This is a case control study involving 30 subjects with 15 cases of RA and 15 control age and sex matched healthy subjects. Recently diagnosed RA patients are included and long term treated RA patients are excluded. Peripheral blood analysis was done in both cases and controls by isolating the miRNA, followed by c-DNA synthesis by reverse transcription. Quantification of the miRNA-24, miRNA 125a-5p & miRNA 132 expression in them by real time PCR was done. Statistical analysis was done by Mann Whitney U test and Spearman correlation to evaluate the statistical significance of this biomarker in early detection of RA.

RESULTS: The average value of miRNA-24, miRNA-125a-5p and miRNA-132 among the patients recently diagnosed with Rheumatoid Arthritis were 0.745, 1.907 and 0.498 respectively. The average value of miRNA-24, miRNA-125a-5p and miRNA-132 among the healthy controls were 2.548, 0.676 and 0.628 respectively. There were significant differences ($p<0.001$) in all three values (miRNA-24, miRNA-125a-5p and miRNA-132) among the two patient groups.

CONCLUSION: The miRNA-24 values were significantly lower in patients with rheumatoid arthritis when compared healthy controls. The miRNA-125a-5p were significantly higher in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-132 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls. This variation in expression profiling and potential role of miRNAs in pathogenesis of rheumatoid arthritis, makes it a potential biomarker for early detection, thereby reducing morbidity and mortality.

KEYWORDS : Rheumatoid Arthritis, miRNA expression profile, Biomarker, miRNA-24, miRNA 125a-5p, miRNA 132

INTRODUCTION:

Rheumatoid arthritis (RA) is the most common autoimmune disorder, with an incidence of about 0.2% - 0.5% in the global population [1]. Nearly about 7 million people are affected with rheumatoid arthritis in India [2]. It is a chronic inflammatory condition with increasing rates of morbidity and mortality [3]. However, the etiology behind this autoimmune disease is unknown in most cases [2, 4]. The clinical course of RA fluctuates and prognosis is unpredictable. In the long term, major outcomes include joint deformity and misalignment, need for joint replacement surgery, functional disability, and premature death due to accelerated atherosclerotic cardio-vascular and coronary heart disease [5].

Currently the diagnosis for rheumatoid arthritis is based on the 'The American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) criteria 2010, which ultimately aims at early detection of RA [6]. However, this criterion only gets fulfilled when nearly more than ten joints are affected, thus giving rise to false negative results in the earlier phases [7]. Early detection and early intervention tends to have better prognosis compared to delayed diagnosis [6,7]. Thus new category of biomarkers which enables early detection is essential.

MicroRNAs (miRNA) are endogenous 20 -22 nucleotide, long non-coding RNAs which regulates gene expression by degradation of mRNA and translational inhibition, post-transcriptionally [8]. They play a very important role in the pathogenesis of many diseases including cancer, cardiovascular diseases and immunological disorders [8,9].

miRNAs are involved directly in modifying the levels of certain regulatory proteins which are essential for normal development and functioning of the immune system. These regulatory proteins have been associated with immunological disorders, in which the miRNAs are found to be mutated or their expression levels dysregulated, consequently triggering altered or impaired function [10].

The aim of our current study is to observe the variations in the expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in peripheral blood mononuclear cells of HC, and recently diagnosed RA patients.

MATERIALS & METHODS:

This is a multicentric case control study conducted from April 2018-September 2018 involving three institutions namely Government Omandurar Medical College and Hospital, Chennai; Institute of Social Obstetrics and Government Kasturba Gandhi Hospital, Chennai & Tamil Nadu Government Multi Super Speciality Hospital, Chennai.

INCLUSION CRITERIA:

CASE GROUP: 15 patients with Rheumatoid arthritis.

1. 30 - 60 years of age
2. The patient should be recently diagnosed with rheumatoid arthritis according to ACR and EULAR criteria.

CONTROL GROUP: 15, age and sex matched healthy individuals

EXCLUSION CRITERIA:

- Patients with long term treatment for rheumatoid arthritis
- Other autoimmune conditions
- Systemic inflammatory conditions and other systemic diseases
- Malignancies
- Cardiovascular diseases

PROCEDURE:

10 ml of venous blood collected with EDTA-2K containing tubes and centrifuged at 400g for 30-40 minutes and stored at -20 degrees Celsius until analysis.

1. **ISOLATION OF MONONUCLEAR CELLS FROM HUMAN PERIPHERAL BLOOD BY DENSITY GRADIENT CENTRIFUGATION:**

Peripheral blood mononuclear cells (PBMCs) are isolated using Ficol-Paque and a Lecuosep tube according to the manufacturer's protocol. The peripheral blood or buffy coat should not be older than 8 hours and should be supplemented with anticoagulants (e.g. heparin, EDTA, citrate, ACD-A, or citrate phosphate dextrose (CPD)).

2.miRNA ISOLATION [38]:

The miRNA is isolated by using mirVanaTM miRNA Isolation Kit following manufacturer's protocol.

1. **CELL LYSIS:** Thoroughly disrupt up to 30 mg of sample material in 300 µL Buffer ML using mechanical devices. Incubate 5 min at room temperature (18–25 °C).
2. **HOMOGENIZATION OF THE LYSATE:** Place a NucleoSpin® Filter Column (violet ring) into a Collection Tube (2 mL, lid). Load the lysate and centrifuge for 1 min at 11,000 x *g* to reduce viscosity and to clear the lysate from undissolved debris. Discard the NucleoSpin® Filter Column (violet ring) and proceed with the flow through.
3. **ADJUST BINDING CONDITIONS FOR LARGE RNA AND DNA**: Add exactly 150µL 96-100% ethanol to 300µL flow through from step 2. Vortex immediately for 5s. Incubate for 5 min at room temperature (18–25 °C).
4. **BIND LARGE RND AND DNA:** Combine a NucleoSpin® Collection Tube (2 mL, lid) and load the sample including RNAA ColRumn (blue ring) with an any precipitate into the column Centrifuge for 1 min at 11,000 x *g*. Keep both the NucleoSpin® RNA Column with bound large RNA and DNA and the flow through containing small RNA and proteins. Place the NucleoSpin® RNA Column in a new Collection Tube (2 mL) without lid. Close the lid of the Collection Tube (2 mL, lid) with the saved flow through. Proceed with the NucleoSpin® RNA Column.
5. **DESALT SILICA MEMBRANE:** Add 350 µL Buffer MDB to the NucleoSpin® RNA Column (blue ring) and centrifuge for 1min at 11,000 x *g*. Discard flow through and place the columnback into the collectiontube.
6. **DIGEST DNA:** Add 100 µL rDNase directly onto the silica membrane of the NucleoSpin® RNA Column (blue ring). Incubate at room temperature (18–25°C) until steps 7–10 are completed, but at least 15 min.
7. **PRECIPITATE PROTEIN:** Add 300 µL Buffer MP to the saved flow through of step4. Vortex for 5s. Centrifuge for 3 min at 11,000 x *g* to pellet protein.
8. **REMOVE RESIDUAL DEBRIS:** Place a NucleoSpin® Protein Removal Column (white ring) in a Collection Tube (2 mL, lid) and load the supernatant from step 7 into the column. Centrifuge for 1min at 11,000x *g*. Discard the NucleoSpin® Protein Removal Column and keep the flow through.
9. **ADJUST BINDING CONDITIONS FOR SMALL RNA:** Add 800 µL Buffer MX to the flow through. Vortex for 5 s.
10. **BIND SMALL RNA:** Load 600 µL of the mixture from step 9 into the corresponding NucleoSpin® RNA Column (blue ring) already containing the large RNA from step 4. Centrifuge for 30 s at 11,000 x *g*. Discard the flow through and place the column back into the collection tube. Repeat this step two times to load the remaining sample.
11. **WASH AND DRY SILICA MEMBRANE:**

1st WASH: Add 600 µL Buffer MW1 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x *g*. Discard flow through and place the column back into the collection tube.

2nd WASH: Add 700 µL Buffer MW2 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x *g*. Discard flow through and place the column back into the collection tube.

3rd WASH: Add 250 µL Buffer MW2 to the NucleoSpin® RNA Column. Centrifuge for 2 min at 11,000 x *g* to dry the silica membrane. Discard Collection Tube and place the NucleoSpin® RNA Column into a new Collection Tube (1.5 mL, lid).

12. **ELUTE RNA:** Add 30µL (high concentration), 50µL (medium concentration and yield) or 100µL (high yield) RNase-free H_2O to the NucleoSpin® RNA Column. Incubate for 1 min at room temperature (18–25 °C). Centrifuge for 30 s at 11,000 x *g*.

3. REVERSE TRANSCRIPTION [39]:

Reverse transcription is done by using Taqman miRNA cDNA synthesis kit according to manufacturer's protocol.

In a RNase-Free 0.2 ml tube, combine the following reagents and preferably in a thermal cycler, incubate the tube for 1 hour at 37degree C, then terminate at 85-degree C for 5 min to inactivate the enzymes. Add 90 microL ddH2O to bring the total volume to 100 microL.

REAGENTS	VOLUME (microL)
mRQ Buffer (2x)	5
RNA Sample (0.25-8 microg)	3.75
mRQ Enzyme	1.25
Total Volume	10

4. miRNA QUANTITATION BY REAL TIME PCR [39]:

Real-time polymerase chain reaction (PCR) is carried out on an Applied Bio Systems 7300 Real-Time PCR System.

A. Choosing miRNA-specific 5'-primer for qPCR:

Primers for miRNA-24, miRNA-125a-5p and miRNA-132 are to be obtained from Applied Bio Systems. 3' primer was supplied with the kit.

miRNA	PRIMER SEQUENCE
miRNA-132	TAACAGTCTACAGCCATGGTCG
miRNA-125a-5p	TCCCTGAGACCCTTTAACCTGTGA
miRNA-24	TGGCTCAGTTCAGCAGGAACAG

B. Quantifying miRNA by qPCR:

Prepare all sample, U6, and standard curve reactions in duplicate. Include appropriate no template controls (NTC) for each primer set.

The data were entered in Microsoft excel and analysed using Statistical Package for Social Sciences (SPSS v16). The numerical variables such as age, height, weight, body mass index and miRNA values were summarized as mean and standard deviation. Frequency and Percentage was used to summarize categorical variables such as age category, gender and BMI category. One-way Analysis of variance (ANOVA) was used to find the significant difference in the socio-demographic and anthropometric characteristics among the patient groups. Mann Whitney U test, a non-parametric test was used to detect differences in miRNA-24, miRNA-125a-5p and miRNA-132 values among the patient and gender groups. Spearman's correlation was used to correlate the miRNA values with age and BMI values. P value of less than 0.05 was considered significant.

RESULTS:

A total of 30 patients were selected equally (15 each) in two groups (Rheumatoid arthritis, and healthy controls). Among the patients, more than half (55.6%) were in the age group of 31-45 years followed by around 45% in the age group 46-60 years (Table 1). Majority of them were females (69%) and around 31% were males. Around 47% and 11% of the patients were overweight and obese respectively.

Table 1: Age, gender and BMI distribution in in healthy controls, OA and recently diagnosed RA patients

Variables	Patient groups Mean (±SD)		
	Rheumatoid arthritis (n=15)	Healthy Controls (n=15)	P value *
Age	43.6 (±8.11)	40 (±6.95)	0.091
Height	152.9 (±10.25)	157 (±11.77)	0.142
Weight	59.2 (±11.02)	62.1 (±10.6)	0.547
Body mass Index	25.2 (±3.38)	25.2 (±3.71)	0.731

***P value by one-way ANOVA**

Table 1 shows the age, height, weight and body mass index distribution among the three patient groups. One-way Analysis of variance (ANOVA) was used to find the significant difference in the socio-demographic and anthropometric characteristics among the three patient groups. There was no significant difference (p>0.05) in age, height, weight and body mass index. The above table proves that there is no bias in selection of patients among the 2 groups with respect to age, height, weight and body mass index.

Table 2: Expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in Rheumatoid Arthritis patients (Objective 1) (N=15)

Expression Profiling	Mean	Standard deviation
miRNA-24	0.745	0.767
miRNA-125a-5p	1.907	0.350
miRNA-132	0.498	0.382

The average value of miRNA-24, miRNA-125a-5p and miRNA-132 among the patients recently diagnosed with Rheumatoid Arthritis were 0.745, 1.907 and 0.498 respectively. (Table 2).

Table 3: Expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in age and gender matched healthy controls (Objective 3) (N=15)

Expression Profiling	Mean	Standard deviation
miRNA-24	2.548	0.598
miRNA-125a-5p	0.676	0.322
miRNA-132	0.628	0.378

The average value of miRNA-24, miRNA-125a-5p and miRNA-132 among the healthy controls were 2.548, 0.676 and 0.628 respectively. (Table 3)

Figure 1: Expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in recently diagnosed RA and health controls

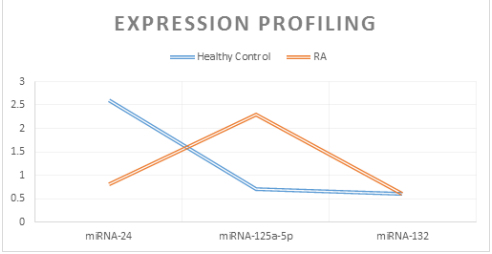

Table 5: Comparisons of expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in recently diagnosed RA, and healthy controls

Expression Profiling	Patient groups, mean (±SD)		p value *
	Rheumatoid arthritis (n=15)	Healthy Controls (n=15)	
miRNA-24	0.745 (±0.767)	2.548 (±0.598)	<0.001
miRNA-125a-5p	1.907 (±0.350)	0.696 (±0.322)	<0.001
miRNA-132	0.498 (±0.382)	0.628 (±0.378)	<0.001

***P value by Mann-Whitney U test as data was not normally distributed**

Figure 1 &Table 5 shows the comparison of expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 in recently diagnosed RA, and healthy controls. Mann Whitney U test, a non-parametric test was used to detect differences in miRNA-24, miRNA-125a-5p and miRNA-132 values among the patient groups (Table 5).

There were significant differences (p<0.001) in all three values (miRNA-24, miRNA-125a-5p and miRNA-132) among the two patient groups (recently diagnosed RA, and healthy controls). The miRNA-24 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-125a-5p were significantly higher in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-132 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls.

Table 6: Correlation between expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 and age of patients

Expression Profiling	Age	p value *
	Correlation Co-efficient	
miRNA-24	0.051	0.744
miRNA-125a-5p	-0.115	0.453
miRNA-132	0.350	0.019

*Correlation co-efficient & p value by Spearman Correlation as data was not normally distributed

Table 6 shows the correlation between expression profiling of miRNA-24, miRNA-125a-5p, miRNA-132 and age. Spearman correlation was used to find significant correlations between miRNA values and age. Only the miRNA-132values significantly correlated with age (p=0.019). Higher age predicted higher miRNA-132 values (positive correlation).

Table 7: Comparisons of expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 among the gender groups

Expression Profiling	Gender groups Mean (±SD)		p value *
	Male (n=14)	Female (n=31)	
miRNA-24	2.316 (±0.761)	1.744 (±1.117)	0.091
miRNA-125a-5p	0.711 (±0.359)	1.265 (±0.718)	0.009
miRNA-132	1.559 (±1.407)	1.349 (±1.313)	0.629

***P value by Mann-Whitney U test as data was not normally distributed**

Table 7 shows the comparison of expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 among the gender groups. Mann Whitney U test, a non-parametric test was used to detect differences in miRNA-24, miRNA-125a-5p and miRNA-132 values among the gender groups. Only the miRNA-125a-5p expression profiles were significantly associated (p=0.009) with gender. The values were higher in females when compared to males.

Table 8: Correlation between expression profiling of miRNA-24, miRNA-125a-5p and miRNA-132 and Body Mass Index

Expression Profiling	Body Mass Index	p value *
	Correlation Co-efficient	
miRNA-24	0.102	0.508
miRNA-125a-5p	-0.072	0.636
miRNA-132	0.182	0.232

**Correlation co-efficient & p value by Spearman Correlation as data was not normally distributed*

Table 8 shows the correlation between expression profiling of miRNA-24, miRNA-125a-5p, miRNA-132 and body mass index. Spearman correlation was used to find significant correlations between miRNA values and body mass index. None of the miRNA values significantly correlated with body mass index.

DISCUSSION:

Currently the diagnosis for rheumatoid arthritis is based on the 'The American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) criteria 2010', which ultimately aims at early detection of RA [6].

However, this criterion only gets fulfilled when nearly more than ten joints are affected, thus giving rise to false negative results in the earlier phases [6]. Early detection and early intervention tends to have better prognosis compared to delayed diagnosis [5, 6]. Thus new category of biomarkers which enables early detection is essential.

miRNAs are a group of approximately 20 to 22 nucleotides noncoding RNAs, which have been proved to cause regulation of gene expression at the post- transcriptional level [8]. The pivotal role of miRNAs in the pathophysiology immune and inflammatory responses is well reported [4]. miRNAs have unique and specific expression profiles in various cells of the innate and adaptive immunity and have crucial roles in the regulation of both cell development and function.

Moreover, when miRNAs are abnormally expressed they can lead to pathological conditions involving the immune system, such as cancer and autoimmunity; they have also been reported to be useful as diagnostic and prognostic markers of both disease type and severity [10].

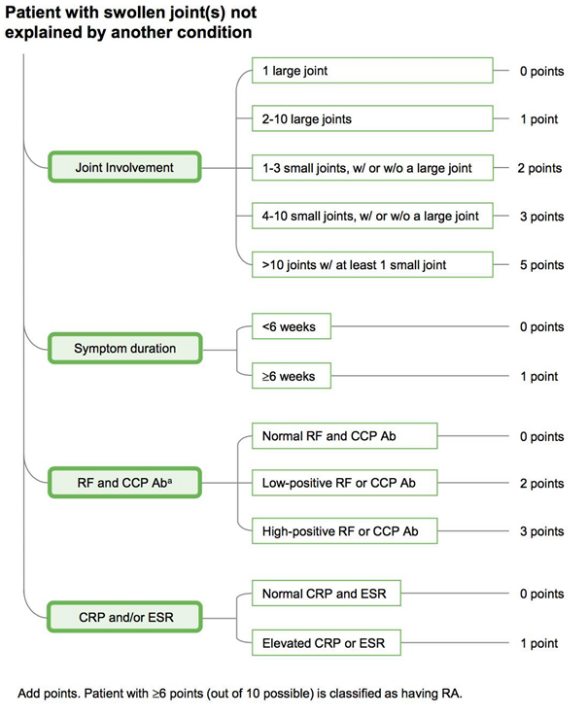

Figure 2: ACR/EULAR Criteria for Diagnosis of Rheumatoid arthritis [6]

In recent years, the pivotal role of miRNAs in the pathogenesis of RA has become the main stream of various studies [32]. miRNAs have been indicated to play an important role in inflammatory responses, synoviocytes proliferation, and production of matrix metalloproteinase in rheumatoid joints [33]. miRNAs play an important role in the development of RA synovial phenotype including hyperplasia of the synovium and joint destruction [4].

miR-146a has been well reported in the regulation of immune and inflammatory responses, and further it has been found that it is strongly expressed in synovial tissue, synoviocytes, PBMCs, and other IL-17 expressing cells from patients with Rheumatoid arthritis [34-37].

Koche Murata et al. used a TaqMan miRNA array to evaluate the expression of 768 miRNAs in plasma from 102 Rheumatoid arthritis patients and 104 healthy controls; they reported 26 miRNAs with significant differences in expression profiling [7]. Dysregulation of miRNAs in T lymphocytes, peripheral blood mononuclear cells, synovial fibroblasts and osteoclasts, each of which is considered to be key cells in joint destruction, have been reported to contribute to inflammation, degradation of extracellular matrix, and invading nature of resident cells. They also concluded that they have identified an increase in the concentration of miR-24, and miR-125a-5p and thus can be used as potential diagnostic markers of RA [7]. Koche Murata et al has also concluded that miRNA-132 was also significantly increased in patients with rheumatoid arthritis and osteoarthritis.

In our study we observed the variation ion expression profiling miRNA-24, miRNA-125a-5p and miRNA-132 in peripheral blood mononuclear cells among 15 newly diagnosed rheumatoid arthritis patients, and 15 healthy controls in a tertiary care centre in India which slightly varies from the results of the study by Koichi Murata et al, which concluded that miRNA-132 is indicatively higher in OA and RA patients and levels of miRNA-24 and miRNA-125a-5p were lower in healthy controls (HC) and OA patients than in RA patients [7, 11]. But the difference is in the population and that it was done using patient's plasma, since the yield of miRNAs from plasma is significantly less in comparison with the study of the same parameter in peripheral blood.

In our study we observed that the miRNA-24 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-125a-5p were significantly higher in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-132 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls.

Maria Cristina Moran-Moguel et al has established the potential of miRNA becoming early biomarkers for rheumatoid arthritis and osteoarthritis by reviewing all the studies under the heading miRNAs and Rheumatoid arthritis [40]. Our study has also shown that there are specific miRNA which are either up-regulated or down-regulated in each groups.

This variation in the levels of miRNAs may lead to induction of pro-inflammatory mediators as quoted by N. M. Baulina et al [41]. N. M. Baulina et al further establishes the relationship between miRNA-125a-5p with the induction of inflammatory pathway mediated by STAT-3. Our study has found that miRNA 125a-5p is significantly increased in rheumatoid arthritis when compared to healthy controls. Masuko Katoh et al have quoted miRNA 125a-5p induces STAT-3 induced WNT5A signalling in the pathogenesis of rheumatoid arthritis [42]. Hence this could be the probable association between miRNAs and the pathogenesis of Rheumatoid arthritis.

This study shows that miRNA-125a-5p is significantly higher in rheumatoid arthritis when compared to healthy controls. But miRNA-24 is lower in patients with rheumatoid arthritis when compared to other groups. The miRNA-132 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls.

Because of this variation in expression and it significant role in pathogenesis of rheumatoid arthritis, miRNAs can be termed as potential biomarkers for early detection. Early detection and early intervention tends to have better prognosis compared to delayed diagnosis and reduces morbidity.

However, its specificity and consistency under different physiological and pathological scenarios is not yet evident. Further there is conflicting information in miRNA expression profiling in different tissues and there is only a limited number of miRNAs identified was dysregulated in the periphery that are capable of differentiating rheumatoid arthritis and osteoarthritis.

Hence our study is a pointer towards using miRNAs as early biomarkers for rheumatoid arthritis. We would recommend studies in this path for the clarification of miRNA participation as biomarkers and for setting up specific guidelines.

The fold change value for each of the miRNAs was calculated by delta-delta Ct method. Statistical analysis was done using Statistical Package for Social Sciences (SPSS v16). Significant differences in the socio-demographic and anthropometric characteristics among the three patient groups was done using One-way Analysis of variants (ANOVA). Mann Whitney U test, a non-parametric test was used to detect differences in miRNA-24, miRNA-125a-5p and miRNA-132 values among the patient and gender groups. Spearman's correlation was used to correlate the miRNA values with age and BMI values.

CONCLUSION:

The miRNA-24 values were significantly lower in patients with rheumatoid arthritis when compared healthy controls. The miRNA-125a-5p were significantly higher in patients with rheumatoid arthritis when compared to healthy controls. The miRNA-132 values were significantly lower in patients with rheumatoid arthritis when compared to healthy controls.

This variation in expression profiling and potential role of miRNAs in pathogenesis of rheumatoid arthritis, makes it a potential biomarker for early detection, thereby reducing morbidity and mortality.

This study is the authentic work of the authors. No financial benefits were received from any commercial party for this study.

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