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PBMC miRNA in diagnosis of rheumatoid arthritis: A miRNA expression analysis study

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***Abstract*---**Background and objective: RA is an autoimmune disorder mediated by autoantibodies. miRNAs have been shown to negatively regulate protein translation via an antisense RNA-RNA interaction. Altered miRNA expression has been well established in autoimmune diseases. In this study, the expression of four microRNA was analysed. Also, correlation between microRNA and immunological markers was examined. Method: RNA was isolated from PBMC obtained from 100 RA patients and age-gender matched healthy controls. Levels of mature miRNA of miR146a, miR155a, miR499 and miR124a were detected using real time PCR. Downstream cytokine was analysed using ELISA from R&D systems. Result: Real time PCR reveals that the expression of miR-146a, miR-155a, miR-499 in PBMC

was found to be down regulated in RA patients when compared to the controls by fold change of 0.15, 0.69 and 0.04 respectively whereas, expression of miR124a was found to be low to detect. Expression of TNF- alpha upregulated (p-value=0.09) and IL6 was found to be insignificantly down regulated in patient. miRNA 155a was found to be in significant negative correlation with rheumatoid factor (r= -0.210; p=0.038). Although we were not able to find any direct with disease activity score. Conclusion and Interpretation: miR146a, miR155a expression level can be important biomarkers for RA disease progression but their values can be affected by treatment modality.

***Keywords*---**rheumatoid arthritis, microrna, cytokine, downregulation, real time pcr, disease activity score.

Introduction

Rheumatoid Arthritis (RA) is the most common autoimmune disease [1] mediated by the presence of autoantibodies [2] and inflammatory markers[3]. It has prevalence of 0.5% to 1% worldwide [4] and characterized by its bilaterally, progressive and destructive nature [5]. RA can cause permanent deformity [6], loss of productivity and decline of quality of life [7]. There is an urgent need to establish diagnostic markers for early diagnosis of RA so that patients are started on treatment as early as possible. Therefore, early diagnosis is the key for successful management of RA. Substantial evidence indicates that microRNAs (miRNA) are aberrantly expressed in inflamed synovium or circulating immune cells of patients. Analysis of miRNA may therefore provide novel avenues for development of molecular diagnostic markers for RA. miRNA analysis may also help to decipher mechanisms which lead to breakdown of tolerance and development of autoimmunity in RA. MicroRNAs (miRNA) are 19-22 nucleotide long, single stranded, non-coding RNA and act as post transcriptional regulators of gene expression. [8].The bind target messenger RNA directly and form base pair guided silencing complex. More than thousand miRNAs representing ~0.0001% of the total genome, play a critical role in the regulation of 10–60% of protein-encoding genes. It includes genes involved immune cell lineage commitment, differentiation and maturation; maintenance of immune homeostasis and normal function [8][9]. miRNAs are expressed on cells of the immune system and influence the molecular pathways that control the development and function of innate and adaptive immune responses[10]. MicroRNAs can be overexpressed or under expressed in various autoimmune diseases MicroRNAs also modulate the differentiation and function of T and B cells [11]. In the present study, we hypothesized that four microRNAs miR-146a, miR-155a, miR-499 and miR124a which play a key role in inflammation mediated immune response may be a marker of disease status in RA.

Method

This study was an open-labeled, cross-sectional and retrospective study. It was based on the descriptive and experimental research methodology. Subject recruitment: A hundred patients with confirmed rheumatoid Arthritis (RA; test

population) from joint clinic and hundred age and gender matched controls were recruited after getting informed consent between May 2016 to Dec 2018. Patients were classified as definite RA by rheumatologists based on American College of Rheumatology-2010 criteria [12]. Demographic data was collected in one-to-one interviews with the study subjects. Inclusion-Exclusion criteria: (1) The patient with the age of >18 years and should have definite RA with the history of symptoms and should be >=6weeks old. (2) there must be evidence of currently active clinical synovitis (i.e., swelling) in at least 1 joint and not better explained by another diagnosis. Sample Collection: Whole blood sample was collected from the patient into EDTA vacutainer (Ref 418354ELV) and plain vacutainer (Ref 418354ELV). Serum was separated from plain vacutainer blood by centrifugation at 4000rpm for 10 minutes. EDTA blood was subjected to RNA extraction.

RNA extraction: Total RNA was isolated from PBMC using miRNeasy mini kit (Cat No. 217004) from Qiagen by using manufacturer instructions. RNA yield was quantified using BioPhotometer plus from Eppendorf. cDNA preparation and Real time PCR: cDNA was prepared from the extracted RNA using Taqman® microrna RT kit from Thermo-fisher as per given instruction. In brief, 7.5ng of total RNA mixed with 5X RT primer, 100mM dNTPs, 1µl of Reverse Transcriptase, 10X Reverse Transcription buffer, 200U/µl RNase inhibitor and DNase-RNase free water to make up the volume of 7µl. cDNA was synthesized in 65 minutes using Thermocycler (vapo.protect) from Eppendorf. Real Time PCR (qRT-PCR) was performed using Taqman® MicroRNA Assays from applied biosystems for mature miRNA (hsa-miR-146-5p (miR-146a); hsa-miR-155-5p(miR-155a); hsa-miR-499-5p(miR-499a) and RUN6B as housekeeping gene, on RGQ from Qiagen as per manufacturer protocol.

To summarize, qPCR was conducted in triplicates of 10µl reaction mix where 1µl of RT reaction product was mixed with 20X Taqman primer, 2X Taqman Universal PCR master mix II and DNase-RNase free water. PCR cycle: 2min at 50^0C, 10 min at 95^0C followed by 40 cycles of 95^0C for 15 sec, 60^0C for 60 sec. Cytokine detection: Serum levels of cytokines, Interleukine-6(IL6) and Tumor Necrotic Factor alpha (TNF-α) were measured by Enzyme Linked Immunosorbent Assay (ELISA). Human IL6 DuoSet ELISA (Cat No. DY206-05) and Human TNF-α DuoSet ELISA (Cat no. DY207-05) from R&D systems were used as per the manufacturer's instruction. Optical density was measured at 450nm and 540nm (reference wavelength) using PR4100 (BioRad) ELISA reader. Data was visualized in Magellan software (V 7.0). Auto antibody detection: Serum levels of RF and CRP were measured using Immunoturbidimetric assay and Particle enhanced immunoturbidimetric assay respectively using cobas c311 automated system. Anti CCP was analysed using Elecsys 2010 electro-chemiluminescence immunoassay (ECLIA). Erythrocyte Sedimentation Rate (ESR) was measured in whole blood by the westergine method. DAS-28-ESR calculator was used for the estimation of disease activity score [13]. Cut-off values for various marker were as follows: RF:<=14 IU/ml; Anti-CCP: <=17 IU/ml; CRP: <5mg/dl; ESR: 0-20mm/hr (female) and 0-15mm/hr (male). Software: Real Time PCR were analysed using software Rotor-Gene Q series 2.3.4 and Bar chart was prepared using GraphPad prism (version 8.0.4).

Statistical analysis, box plots and Receiver Operating Characteristic curve (ROC curve) were performed using IBM SPSS version 26. Statistical Analysis: Statistical summaries for continuous data were presented as mean ± SD or Median (Range) as per the distribution of data. Summary of categorical data presented in frequency and percentage. Bar chart and box plots were presented for data visualization. Karl-Pearson correlation coefficient was used to find association between two numbers. Categorical and continuous data were analysed by using chi-square and T-test respectively. Statistical significance is assessed considering Bonferroni's correction for multiple tests.

Results

Average and median age of participants in the patient group was 48.02±10.7 and 49.50 (range: 21-78 years), and in the control group was 45.44±10.5 and 43 (range: 18-71 years). Both groups have 84% female and 16% male. Recruited population was statistically identical with a p-value of 0.087. Demographic details of patients and clinical data of recruited of recruited population are shown in table I. The peak of onset of RA disease was obtained in two age groups, 31-40 years (n=35) and 41-50 years (n=30). The median disease history was 5 years (range: 6 months -15 years).

MicroRNA analysis

Expression of four microRNA was studied. Interim study analysis of 20 samples, showed that expression of miR124a was undetectable in both populations. Expression Analysisanalysis obtained from software REST (version 2.0) is shown in fig 1 (a-c). Therefore, miR124 was dropped for further detection. The median (IQR) levels of miR-146a, miR-499a and miR155a in 100 patients were 29.55(273.51), 0.02(0.12) and 13.12(71.51), respectively. The median (IQR) levels of miR-146a, miR-499a and miR155a in 100 controls were 530.2(1962.9), 0.07(0.14), 71.5(196.4) respectively. Distribution of microRNA expression is shown in fig 2 (a-c)**.** Fold change ($2^{-\Delta\Delta Ct}$) of miR-146a, miR-499a and miR155a microRNAs in patients as compared to controls was 0.15, 0.04 and 0.69 respectively. Expression of miR-146a, miR-499 and miR155a was significantly down regulated (p-value: 0.000, 0.003 and 0.000 respectively) in patient population as compared to control.

Cytokine Analysis

Median (IQR) values of IL-6 cytokine were 7.89 (0.00 to 364.6) in the patient group and 5.06 (0.00 to 384.2) in the control group**.** Further, median value for TNF-α expression was 4.7 (0.00 to 1100.10) in the patient group and 8.04(0.00 to 749.99) in the control group. Expression of IL6 found to be downregulated in RA patients in comparison to control, (p-value=0.46), whereas, Expression of TNF-α was upregulated in patients' population (p-value=0.09). Distribution of cytokines is shown in fig 2 (d-e). Correlation between microRNA, Cytokine, and disease markers: A moderate but significant correlation was observed between miR-146a and miR155a (r=0.613; p=0.000), miR155a and miR499 (r=0.608; p=0.000), miR146a and miR499(r=0.609; p=0.000). Correlation between IL-6 and TNF-α was found to be r=0.298; p=0.003. Correlation between microRNA expression,

cytokine level and another immunological marker was calculated. miR-155a showed negative correlation with RF (r= -0.210; p=0.038). Correlation between immunological markers and DAS-28 has been shown in fig 3(a-i).

ROC curve analysis

We performed ROC curve analyses and calculated the AUC to assess the discriminatory ability of three miRNAs in patient and control population. On ROC curve analysis of all three miRNAs showed a good AUC: miR146a (AUC 0.722, 95% CI 0.652-0.793, p<0.0001), miR155a (AUC 0.667, 95% CI 0.591-0.742, p<0.0001), miR499 (AUC 0.620, 95% CI 0.540-0.700, p=0.003). We found sensitivity and specificity of miR146a (88%, 50%); miR155a (60%, 70%) and miR499(68%, 64%). Pairwise comparison of ROC curves for these microRNA revealed that miR146a is more significant than miR155 (p=0.02) and miR499(p<0.01) whereas the difference between miR155a and miR499 is non-significant (p=0.27). Fig 4 (A-F) shows ROC curve for three microRNA and pairwise comparison of microRNA.

Discussion

Rheumatoid arthritis is a pathogenic inflammatory condition, which mainly involves body joints. Early and sensitive markers are required to evaluate the activation of the immune system and relate it to its pathogenesis as well as selecting the most suitable therapy for each patient. CRP, RA factor and anti CCP in plasma are used to make diagnoses of RA but they are not very specific to RA during the early phase. In absence of specific biomarkers, many cases of rheumatoid arthritis remain undiagnosed during early periods of disease progression. There is an immense need to find better and efficient biomarkers for early diagnosis of RA.

miRNAs are pleiotropic; a single miRNA can regulate a cellular response by targeting multiple components of a biological pathway [14]. It can play a central role in regulating many biological processes, like development, differentiation, apoptosis and survival. They are also involved in modulating the innate and adaptive immune response [15]. Circulating miRNA in plasma have been proposed as biomarkers owing to tissue specificity and high release kinetics. miR-146a, miR155a and miR-499 are the most studied microRNA. They are present in many biofluid and tissue sites such as synovial fluid, whole blood, PBMC, synovial tissue etc. Our study aims to evaluate plasma levels of miRNA 146, miRNA 155, miRNA 499 and miRNA 129 in the RA patient population in comparison to the control group.

We observed downregulation of miRNA 146a and miRNA 155 in the RA patient population with 88% and 60% sensitivity respectively. The inhibition of miRNA-146a has been reported to increase inflammatory marker IL-1β, IL-6 and TNF-α secretion [16]. It is a known fact that inflammatory markers rise in case of RA. Konstantin D. Taganov has also proposed a role for miR-146 in control of Toll-like receptor and cytokine signalling through a negative feedback regulation loop [17]. It has been reported that miRNA-146 inhibits pro-inflammatory cytokine secretion through IRAK1, which indicates that miRNA-146 functions as a negative regulator

of inflammation [16]. Thus, downregulations of miR146a in the RA patient population indicate presence of inflammatory disease. ROC analysis of miR146a shows high sensitivity but low specificity.

miRNA-155 is also a crucial regulator of innate immunity and is expressed in a wide variety of immune cells during the inflammatory response, including macrophages, dendritic cells and several types of lymphocytes. In macrophages, miR155 is substantially up-regulated by Toll-like receptor (TLR) ligands[18][19]. In glioma cells high miR-155 expression has been associated with strong transcriptional changes and NFκB pathway activation [20]. miRNA 155 drives their inflammatory response by epigenetic regulation of mRNA targets that are inhibitors of innate cell activation like phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP-1);(an inhibitor of TLR/PI3/Akt kinase pathways), SOCS-1 (STAT pathway inhibitor), Bcl6 (inhibitor of NF-κB) [21]. Hence, miRNA155 regulates the inflammatory cytokines. But in our test population we observed down regulation of miRNA 155 as compared to control. Study published by Ankita et al. has shown miR-132, miR-146a and miR-155a as potential responsiveness biomarkers for MTX therapy [22]. Methotrexate, nowadays, is considered as the first line of medication of rheumatoid arthritis patients [23]. MTX is known to prevent radiological progression and modulation of cytokine expression [24]. We speculate that down regulation of miRNA 155 may be the effect of Methotrexate (MTX) therapy to the patient. miR-499 is involved in the regulation of genes coding for IL-17RB, IL-23a, IL-2RB, IL-6, and IL-2. These proteins are involved in inflammation signalling pathways[25]. We observed significant downregulation of mi499 when compared to control but their absolute expression level of miRNA499 was very low in our study population.

Further, we observed cytokine levels of IL-6 and TNF alpha. We found that there was suppression of IL6 level and increase in TNF alpha in RA patients but the change was not significant when compared to control. One of the Iranian studies also reported an insignificant increase in TNF alpha level in patients treated for RA as compared to control [26]. Rheumatologists evaluated disease activity using DAS-28 criteria. DAS/DAS28 is a continuous measure of RA disease activity that combines information from swollen joints, tender joints, acute phase response and general health. serum markers concentrations were analysed in relationship to the clinical assessment. Our recruited control and RA populations showed significant differences between expression of immunological markers in the serum. Expression of inflammatory markers (CRP) and autoantibody (RF and Anti-CCP) are significantly high in RA as compared to control. Rise in immunological markers has been reported by many groups [27]. Correlation analysis of DAS-28 with RF, ESR and CRP; found to be in strong correlation. High concentration anti-CCP is considered specific to RA. But, Anti-CCP showed positive correlation with RF only and showed no correlation with disease activity in our study group. Our results are in affirmation with the findings from the study of Forslind et al 2004 [28] [29] . RF factor specificity increases considerably with higher values (RF> 50 IU/mL). Elevated concentrations of RF and CRP were found in about 72% and 51% of the study population respectively.

We also evaluated correlation between miRNA expression levels and immunological markers. But our study found no correlation of miRNA146 and

miRNA155 with any immunological marker. Our study has some drawbacks. Firstly, the presented study is a cross-sectional study where data was collected at a single time point. We have not collected data for change in patient's disease activity and autoantibodies levels during disease progression. Thus, the second limitation is in the context of the study design, as this study was only hospital-based and perhaps cannot be generalized at a community level. Thirdly, recruited patients were on different doses of MTX at the time of data collection.

Conclusion

Our study results suggest that miR146a, miR155a expression level can be important biomarkers in case of RA disease progression. But their level might be affected with the RA treatment regimen. It will be interesting to assess the relationship between miRNA level and RA activity in patients under different drug regimens.

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