REVIEW ARTICLE

Correlation Between miR-223 Expression and Rheumatoid Arthritis

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ABSTRACT

Patients diagnosed with rheumatoid arthritis (RA) show symptoms of joint affiliations. Focusing on the epigenetic factors leading to RA, the expression of several miRNAs are assumed to be one of the components influencing the cause of RA. Consequently, the increased expression of miR-223 is hypothesized to be involved in the pathogenesis of RA. This study aims to analyze the increased expression of miR-223 of RA patients in comparison with the normal and healthy representatives. Five methods from five different studies were involved in this review. The RNA was first isolated from heparinized venous blood or peripheral blood of RA patients and healthy controls. Reverse transcription was done to convert isolated RNA into cDNA and the expression of miR-223 was then measured using real-time PCR. The expression of miR-223 had exhibited a considerable increase in its concentration on RA patients when compared to healthy controls. Besides, the heightened concentration is taken upon consideration as miR-223 has a role in the regulation of the immune system and inflammatory responses.

Keywords: Rheumatoid arthritis; microRNA; miR-223; autoimmunity; inflammation.

INTRODUCTION

Rheumatoid Arthritis (RA) is a disease that attacks the joints caused by a chronic inflammatory autoimmune response. Symptoms including joint pain, stiffness, swelling, or weakness, fatigue, anemia, malaise, lumps, and redness of the skin are often found in RA patients. RA affects 0.24-1% of the human population and it is more common in women than men (England & Mikuls, 2019). Moreover, some important factors that contribute to the pathogenesis of RA include genetics, epigenetics, and the environment. As such, the activity of microRNA (miRNA) is the key major component among the epigenetic regulatory mechanisms (Fulci et al., 2010).

MicroRNAs (or often called miRNAs) are small, non-coding RNA molecules that only consist of 21-24 nucleotides. MicroRNAs are thought to be able to influence genes at the post-transcriptional level either by degrading the mRNA or promoting mRNA translation. Thus, it can be said that miRNA regulates gene expression (Huang et al., 2019; Lu et al., 2014). According to Taïbi et al. (2014), miR-223 was first described to play a role in the differentiation of hematopoietic lineages. In addition, miR-223 is also reported to be essential in the immune system and inflammation responses. Recently, increased levels of miR-223 have been found in RA patients (Fulci et al., 2010). Therefore, the role of miR-223 in the immune system and inflammation responses are suspected to have a major influence on the pathogenesis of rheumatoid arthritis. The aim of this study is to determine the correlation between miR-223 expression and the RA.

METHODOLOGY IN DETERMINATION OF miR-223

A search through Google Scholar with keywords 'Expression of miR-223' and 'Rheumatoid Arthritis' were used to narrow down the data. For methodology specifically, the data used were primary data sources, specifically original research in the form of journal articles.

Patients and Control

A total of five methods from five different studies were used in this review. The following table listed the number of patients and controls, as well as details related to the patients and controls.

Table 1. Data of patients and controls used in the review from five different studies.

Study*	Year	RA Patients**	Age Range	Healthy Control (HC)	Age Range	Notes
Fulci et al.	2010	37	22-82	10	N/A	Out of 37, 28 of RA patients were under drug treatment
Murata et al.	2010	40	22-77	30	32-62	Drug treatments were not specified
Lu et al.	2014	27	N/A	24	N/A	Age and sex-matched for HC Drug treatments were not specified
Khalifa et al.	2016	21	60 ± 12***	22	54.7 ± 6.4***	Age and sex-matched for HC All RA patients were under drug treatment
Taha, Shaker, Abdelsalam, & Taha	2020	120	19-60	30	26-54	Age and sex matched for HC All RA patients were under drug treatment

*All the studies were approved by the respective institutional review board and ethics committee. All studies also obtained written consent from all the participants.
**All the RA patients fulfilled the 1987 American College of Rheumatology revised criteria for the classification of RA.
***Number expressed as mean ± standard deviation.

Isolation of Total RNA

In a study conducted by Lu et al. (2014), total RNA was isolated from heparinized venous blood through peripheral veins that was mixed with 2% dextran solution, which is one-fourth of its volume. The samples were incubated for 30 minutes at room temperature and the supernatants were collected. Afterward, they are added to the Ficoll-Hypaque density gradient solution and centrifuged at 250g for 25 minutes. The T cells were then purified by IMag Cell Separation System using the anti-human CD3-coated magnetic beads and concentration was adjusted accordingly by adding RPMI-1640 medium until reaching a concentration of 1 x 10^6/ml. After that, the miRNAs were extracted using mirVana miRNA isolation kit and the concentration of RNA was measured with the NanoDrop Spectrophotometer.

The following three studies were using RNA extracted from peripheral blood samples, but with different types of cells. A blood collection using EDTA-2K tubes and separation by lymphoprep density gradient centrifugation. It was then followed by the purification of T cells by using kit by Miltenyi Biotec which principle depends on the separation and depletion of non-T cells. However, this particular study did not further describe the RNA extraction and quantification method.

Meanwhile, Khalifa et al. (2016), focused on mononuclear cells in the peripheral blood. The blood was collected with EDTA-2K-containing tubes. Then, by utilizing the ficoll-hypaque density gradient and miRNeasy Mini Kit, mononuclear cells and total RNA were extracted respectively. The concentrations of RNA were then measured by using the NanoDrop Spectrophotometer.

Unlike the other four studies mentioned above, experiments done by Taha, Shaker, Abdelsalam, & Taha (2020) used serum as the sample. However, the RNA extraction steps were more or less the same

Measurement of miRNAs Expression by Real-time PCR

Based on a study conducted by Lu et al. (2014), all of the miRNAs from heparinized venous blood samples were converted to cDNAs by using a one-step reverse transcription. After that, the ABI Prism 7500 Fast Real-Time PCR system was performed to quantify the miRNAs expression levels. In this system, U6 small nuclear RNA (snRNA) was used for normalization. Lastly, the normalized miRNA levels were defined by the equation of 39 – threshold cycle (Ct) after normalization.

Meanwhile, the measurement of miR-223 expression from peripheral blood samples was divided into three methods based on three studies. In research done by Fulci et al. (2010), the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Applied Biosystems. These PCR reactions were performed in triplicate with small RNA U6B as the control for data normalization.

Second, Khalifa et al. (2016) studies study by Fulci et al. (2010) focused on CD3+ lymphocytes cell. The isolation started with

Still using peripheral blood by collecting it with EDTA-2K tube, Murata et al. (2010) focused on the plasma. The samples were first centrifuged at 400g for 7 minutes in order to separate the plasma. To isolate the RNA, 100µl of plasma was thawed on ice, diluted with 150µl of RNase free water, and lysed with 750µl of Isogen LS. Then, 25 fmol of synthetic *C. elegans* miRNA cel-miR-39 were added, homogenized, and incubated for five minutes. After that, the samples were added with 0.2 ml chloroform, shaken vigorously for 15 seconds, and incubated at 4°C for 15 minutes. Finally, the High Pure miRNA Isolation Kit was used to the 300µl of aqueous phase to isolate and purify the RNA.
as the study by Khalifa et al (2016), still using the miRNeasy extraction kit to isolate RNA and NanoDrop Spectrophotometer to measure the RNA concentration.

converted the RNA into cDNA using Multiplex RT Primers and TaqMan MicroRNA Reverse Transcription kit. After that, the cDNA underwent a pre-amplification using homemade MegaplexTM PreAmp Primers and TaqMan® PreAmp Master Mix. Next, the Viia7 real-time PCR system was performed with RNU48 as a control for data normalization. Afterward, the expression of pri-miRNAs encoding for miR-223 was quantified using pri-miRNA Gene expression assays with GAPDH as the endogenous control. Lastly, the miRNA expression level was calculated using the comparative Ct method.

Third, the research conducted by Murata et al. (2010) also performed reverse transcription but with the NCode VILO miRNA cDNA Synthesis Kit. Then, real-time PCR was executed by using the Applied BioSystems 7300 Real-Time PCR System. Afterward, the expression level of miRNA was analyzed with SDS Relative Quantification Software version 1.3.

Lastly, the RNA obtained from serum

samples was converted into cDNA using the miScript II RT Kit in a study done by Taha, Shaker, Abdelsalam, & Taha (2020). After that, the serum expression level of mature miR-223 was evaluated using miScript miRNA PCR primer assays and miScript SYBR green PCR kit with miRNA SNORD68 as the internal control. Next, a real-time PCR method was performed using Rotor gene Q System. Then, the ΔCt and fold changes were calculated for relative quantification.

Statistical Analysis

In this review, the P-value was calculated with five different methods. The following table listed the type of sample with its statistical analysis method.

Table 2. List of statistical analysis methods used in the review from five different studies.

Study	Sample	Statistical Analysis Method*
Lu et al.	Heparinized venous blood (T cell)	Paired or unpaired Mann–Whitney *U*-test
Fulci et al.	Peripheral blood (T cell)	Wilcoxon rank-sum test
Khalifa et al.	Peripheral blood (mononuclear cells)	Pearson R test and Mann-Whitney test
Murata et al.	Peripheral blood (plasma)	Bonferroni method
Taha, Shaker, Abdelsalam, & Taha	Serum	Student's t test or one way ANOVA*

*A *P*-value of less than 0.05 was considered statistically significant.

INCREASED EXPRESSION OF miR-223 DECREASES IGF-1R MEDIATED IL-10 PRODUCTION IN RA PATIENTS

A study by Lu, et al. (2014) has verified that miR-223 expression is significantly higher in RA patients' T cells compared to normal healthy T cells. Subsequent study by transfecting miR-223 mimic into Jurkat cells also revealed a suppression of IGF-1R (Interleukin-like Growth Factor Receptor) protein expression but not accompanied by IGF-1R mRNA suppression. This finding indicates that miR-223 inhibits the translation of IGF-1R protein while not interfering with mRNA expression. Since IGF stimulates the secretion of Th2 cytokine IL-10, Lu, et al. (2014) performed another experiment revealing IGF-1 stimulates IL-10 production in normal healthy T-cells. However, IGF-1 mediated IL-10 production did not happen in RA T-cells. Combining these results together, Lu, et al. (2014) suggested that increased miR-223 expression in RA patients has caused downregulation of IGF-R protein in T cells, thus the T cell cannot exert response towards IGF cytokines. Subsequently, T cells will not produce IL-10, which is an anti-inflammatory cytokine (Koojiman, 2004). IL-10 deficiencies cause imbalance in proinflammatory and antiinflammatory cytokines, leading to inflammation in the joints hence contributing to RA pathogenesis

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OVEREXPRESSION OF miR-223 IN T-LYMPHOCYTES FROM PERIPHERAL BLOOD OF RA PATIENTS

On the other hand, Fulci et al. (2010) has also discovered an upregulated expression of miR-223 in T-lymphocytes from peripheral blood of both treated and

untreated RA patients compared to healthy controls. In addition, the qRT-PCR analysis displayed no significant difference in miR-223 expression between RA patients that were untreated and treated with low-dose corticosteroids. Besides that, Fulci et al. (2010) also compared the expression of miR-223 in a specific subpopulation of T-lymphocytes, which are CD4 and CD8 cells. As a result, the miR-223 expression is found to be predominantly higher in CD4 cells rather than CD8 cells. Hence, Fulci et al. (2010) suggested CD4 cells as the main actor in RA pathogenesis. Other than CD4 cells, a study conducted by Hirota et al. (2007) also suggested that Th17 lymphocytes may play crucial roles in RA pathogenesis similar to CD4 cells. Surprisingly, miR-223 expression was only overexpressed in naive CD4 cells and not in Th17 cells.

In addition, Fulci et al. (2010) also observed whether miR-223 overexpression is associated with T-lymphocyte activation in RA patients. However, the expression of miR-223 was not observed in the Jurkat cell line after TCR stimulation. On the other hand, an inconsistent expression of miR-223 was also detected in naive CD-4 cells of healthy controls even after TCR stimulation. Therefore, all of these results suggested that the overexpression of miR-223 is associated with RA pathology and not T-cells' activation. However, future studies were recommended to identify the function of miR-223 in T-cells and its role in RA pathogenesis.

miR-223 EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Unlike the previous two studies, a study by Khalifa et al. (2016) instead revealed a similar miR-223 expression profile between RA patients and healthy control. Although, a tendency of overexpression was indeed observed in the RA patients. This finding was quite contradictory with studies conducted by Shibuya et al. (2013), also using PBMCs, in which overexpression of miR-223 is observed and associated with osteoclastogenesis. Khalifa et al. (2016) described the reason is due to the sample group used in the experiment consisting of RA patients with mostly low disease activity or remission. Though study by Churov, Oleinik, & Knip (2015) and Fulci et al. (2010) reported otherwise. They described miR-223 expression does not correlate with disease activity, instead with rheumatoid factor. Khalifa et al. (2016) study also demonstrated a significant correlation between miR-223 expression and anti-citrullinated peptide antibodies (ACPA) concentration. Thus suggesting an important role of miR-223 in the RA pathology. Lastly, in this experiment, Khalifa et al. (2016) also established the fact that there was no gender-bias related to the disease, based on the miR-223 expression profile.

INCREASED EXPRESSION OF miR-223 IN PLASMA ACTS AS BIOMARKER IN RA PATIENTS

The study done by Murata et al. (2010) focused on the miRNA produced in plasma of healthy controls and RA patients by measuring the responsible miRNA, in this case, miR-223. The excellent stability of miRNA after freeze thawing allowed an assured observation of miRNA concentration within the plasma. Moreover, plasma miRNA plays a role in determining RA as a clinical biomarker, plasma miR-223 in RA showed a significantly higher value when compared to healthy control. In RA patients, the average concentration of miR-223 present in the plasma was shown to be $1.3x10^3$ pmol/L. Furthermore, it was also found that the plasma miRNAs, including miR-223, are secreted in the formation of exosomes containing mRNAs and miRNAs. These exosomes perform in mechanisms related with RA, hence the underlying source of the disease.

OVEREXPRESSION OF SERUM miR-223 IN RA PATIENTS

Another study by Taha, Shaker, Abdelsalam, & Taha (2020) also confirmed that serum miR-223 expression levels were significantly upregulated in RA patients compared to healthy controls with median fold change of 21.1 (P=0.001). Moreover, miR-

223 was also overexpressed in peripheral blood CD3+ and CD4+ naive T-lymphocytes of RA patients. These results also support the previous study by Fulci et al. (2010) that demonstrated overexpression of miR-223 in blood and synovial T-lymphocytes from RA patients. Taha, Shaker, Abdelsalam, & Taha (2020) therefore concluded that miR-223 plays a role in the RA pathogenesis. They suggested that the positive correlation of miR-223 and RA is due to the miR-223 role in regulating progenitor cell proliferation, granulocyte function, macrophage differentiation, inflammation and immunity. In addition, the upregulation of miR-223 is also associated with subcutaneous nodules presence, which suggests that miR-223 is correlated to RA development and severity. Additional study to evaluate miR-223 as a potential biomarker was also done by using ROC (Receiver Operating Characteristic) analysis. The result of this study is aligned with the previous study by Murata et al. (2010), suggesting that miR-223 could serve as a potential biomarker for RA.

CONCLUSION

In conclusion, an upregulated expression of miR-223 was observed in all samples, including heparinized venous blood (T-cell), peripheral blood (T-cell, mononuclear cell, plasma), and serum. These findings solidify the idea of miR-223 role in RA pathogenesis. Different approaches were used to prove the idea, including the increased IL-10 production, expression in Jurkat cell line after TCR stimulation, ACPA concentration, and the ability of miR-223 in regulating the immune cells. Interestingly, research also suggested the possibility of miR-223 playing part in inflammation of the joints, but not T-cells' activation. The potential of using miRNA expression as a biomarker in determining RA had also been suggested through these findings. However, the exact role of miR-223 in RA pathogenesis is still unclear. Hence, it is suggested to do further validation research, such as miR-223 silencing, to fully confirm the miR-223 roles in RA pathogenesis.

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