

miRNA expression profile in Behcet patients using colchicine

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Abstract

Aim: Behcet's disease (BD) is a multi-systemic disorder which shows inflammatory vasculitis characteristic with recurrent oral aphthae, eye lesions, and genital ulceration. MicroRNA's (miRNAs) are small non-coding RNA molecules that play an important role in the regulation of various biological processes. We aimed to determine miRNAs that are thought to be associated with inflammatory processes in BD patients using colchicine in the active period.

Materials and Methods: We enrolled 24 patients with BD receiving colchicine treatment and a control group comprising 30 healthy unrelated individuals. We studied with a panel where 377 miRNAs were included in the assay.

Results: miR-20a, miR-34a, miR-197, U6snRNA, miR-205, miR-222, miR-296, miR-302a, miR-302c, and miR-372 were observed up-regulated in BD. miR-518b, miR-874 were found down-regulated within the patients. These miRNAs are known to play a role in cancer-related pathways.

Conclusion: In patients treated with colchicine, the change in the expression of these miRNA may give an idea of the effect on the progression of the disease. However, patients in the active period also require further studies to fully understand the role and mechanism of action of the drug.

Keywords: Behcet disease; colchicine; microRNA

INTRODUCTION

Behcet's disease (BD), also called Behcet's syndrome is a chronic disease which shows inflammatory vasculitis characteristic with recurrent oral aphthae, eye lesions, and genital ulceration. Although the etiology of BD is not yet known, it is thought that the immune-mediated reaction triggered by some infectious agents and environmental factors is increasing in people with a genetic predisposition. Recently, an international working group on Behcet's disease revised the criteria for the classification/diagnosis of BD (1). Behcet's disease and auto-inflammatory diseases share several clinical features (2). The reason why the disease is classified as an autoimmune disorder is that it contains positive responses to classical immunosuppressors. Other features that imply the auto-inflammatory role of the disease include clinical periods of recurrent inflammation, predominantly characterized by neutrophils (3), and efficacy in treatments involving anti-inflammatory agents such as colchicine (4).

The anti-inflammatory feature of colchicine affects many mechanisms. The best-known feature of these mechanisms is the potential for colchicine to bind to free tubulin dimers that block subsequent microtubule polymerization when incorporated into microtubules (5). It appears that this mechanism is directly responsible for the effect of colchicine on cytokine release and cell migration, and plays a critical role in collapse of inflammatory cell efficiencies by colchicine (6). Colchicine inhibits microtubule-based inflammatory cell chemotaxis and altering neutrophil deformability (7). Also, colchicine can dose- dependently modulates leukocyte-mediated inflammatory activities, including inhibition of leukocyte superoxide production and the release of various cytokines and pyrogens (8-10).

Recently, the role of microRNAs (miRNA) in the pathogenesis of inflammatory multisystem disease has been the focus of many researchers. Short (about 19-22 nucleotides) single-stranded and non-coding

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miRNAs that bind to specific target mRNAs to regulate gene expression play an important role at different levels on both innate and adaptive immune system cells. For example, they play an important role in granulopoiesis, T cell and B cell development and maturation, antigen presentation. miRNAs have been shown to control many immune processes, including Toll-like receptor signaling cascade and cytokine generation, immunoglobulin class-switch recombination in B cells, and T cell receptor (TCR) signaling (11). There has been widespread discussion about its therapeutic uses for immunological diseases. Indeed, abnormal expression of miRNAs is often seen in human diseases, including inflammatory disorders and autoimmunity (12,13).

Several miRNA profile studies on Behcet's patients have been reported in the literature (14-19). There are a limited number of studies that shown miRNA and colchicine relation. However, the effect of the anti-inflammatory drug on the miRNA expression is not yet known. In this study, gene expression levels of 377 miRNA on BD patients' peripheral blood mononuclear cells (PBMC) were investigated. We identified miRNAs associated with in BD patients using colchicine in the active period.

MATERIALS and METHODS

Sample collecting and RNA isolation

We enrolled 24 (13 female, 11 male) patients with Behcet's Disease receiving care at Ataturk University Training and Research Hospital and a control group comprising 30 (10 female, 20 male) unrelated individuals. The criteria for inclusion in the healthy control group: no clinical signs of BD, no family history of BD, and no active infection or systemic disease. As an anti-inflammatory drug, patients treated with colchicine were selected. The patients were examined according to the International Working Group criteria for BD. Age, gender, major types of BD involvement (oral and genital ulcerations, cutaneous, ocular, and neurological symptoms), HLA-B*51 positive status, pathergy reaction, colchicine dose, duration of use of the drug, family history BD were questioned. The exclusion criteria were as follows other autoimmune diseases, pregnancy and lactation, diabetes, hypertension, severe cardiovascular and cerebral disease, and malignancies.

All subjects gave written consent and the study was approved by the Atatürk University, Faculty of Medicine Ethic committee, 06.10.2011 no:2011.4.1/14 Ataturk University Medicine Faculty Institutional Review Board.

Peripheral Blood samples drawn into EDTA tubes. miRNA was extracted from Peripheral Blood Mononuclear Cell (PBMC) by the High Pure miRNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the protocol provided by the manufacturer. The quantity and quality of the isolated miRNA was determined by OD260 / 280 using a NanoDrop™ 2000 (Thermo Scientific, Worcester, MA).

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed a high-throughput instrument (BioMark, Fluidigm, San Francisco, California) with DynamicArray chips (Fluidigm, San Francisco, California).

cDNA protocol

Each sample consisting of 2.0 µl miRNA was reversely transcribed to cDNA the usage of TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Reaction conditions; observed by means of 40 cycles at 16°C for 120s, 42°C for 60s, 50°C for 1s and subsequently 85°C for five min to inactivate MMLV-RT.

miRNA PreAmplification Protocol

Two µl of cDNA was transferred to a 96 well plate and 8 µl of DNA Suspension Buffer (TE buffer) was added. PCR products were amplified using TaqMan® PreAmp Master Mix and MegaPlex PreAmp Primers (Applied Biosystems, Foster City, CA). Reaction conditions; 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min, followed through 18 cycles of 95 °C for 15s and 60 °C for 4 min and 99.9 °C for 10 min and finally 4 °C for ∞ .

miRNA TaqMan® Dynamic Array Protocol

Two µl of PreAmplified cDNA was transferred to a 96 well plate and 18 µl of DNA Suspension Buffer (TE buffer) was added. PCR products were amplified the usage of the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA) and GE Sample Loading Reagent (BioMark, Fluidigm, San Francisco, California). 3.15 µl diluted PreAmplified cDNA was transferred into each well of 96 well plate which previously 3.85 µl master mix and loading reagent pipetted. 5.0 µl of this master mix and diluted PreAmplified cDNA mixture was transferred into Dynamic Array Sample inlets.

Quantitative PCR analysis

The $2^{-\Delta\Delta CT}$ method is used to analyze relative changes in gene expression from real-time quantitative PCR testing. *RNU44*, *RNU48* genes have been used as a reference gene. The data had been normalized to *RNU44* and *RNU48* expression with a geometrical mean, and a threshold cycle (Ct) cutoff was set to twenty-five cycles.

miRNA expression was analyzed by the comparative CT method:

$$\Delta CT(\text{Control sample}) = CT(\text{control}) - CT(\text{Reference})$$

$$\Delta CT(\text{Behcet sample}) = CT(\text{behcet}) - CT(\text{Reference})$$

$$\Delta\Delta CT = \Delta CT(\text{Behcet sample}) - \Delta CT(\text{Control sample})$$

$$\text{Relative miRNA expression (Expression fold change)} =$$

$$2^{-\Delta\Delta CT}$$

Statistical analysis

The results were statistically analyzed. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) version 20.0 statistical analysis software. Statistical evaluation of miRNAs between Behcet's patients and healthy control groups was compared with Student's t-test $p < 0.05$ and the fold change criterion (FC ≥ 121) were considered statistically significant.

RESULTS

Twenty-four subjects have been evaluated. The average age was 33.5 ± 5.4 years, and the female / male ratio was $14/10 = 1.4$. The average colchicine dose was 1.50 mg/day. Table 1 summarizes the clinical and laboratory data of all study participants.

In this study, 377 miRNA from PBMC was examined, the expression data was normalized. miRNA expressions of patients and controls were shown in Table 2. Up and down regulated miRNA expressions were observed in patients. However, there was no noticeable difference in 64 miRNA expression levels between Behcet patients and controls. In addition, 295 miRNA is not expressed in either group. PBMC and 12 different miRNAs were differently expressed in Behcet's patients. However, this difference is statistically significant, $p < 0.05$, Table 2. Also, Supplementary file 1 presents our results compares with other studies.

Table 1. The clinical features of the subjects

Clinical and laboratory characteristics	BD, n (24)	Control, n (30)
Age; years	33.5±5.4	35.5±4.6
Gender ratio	1.4	0.8
Pathergy test positive	10	
Oral aphthae positive	17	
Skin lesion positive	3	
Pan uveitis positive	13	
Posterior uveitis positive	11	
Genital ulcers	3	
Joint manifestation	1	
Gastrointestinal involvement	0	
Association with HLA-B*51	6	
Duration of use of the drug	2	
Colchicine dose; mg/day	1.50	
Family history of BD	3	

Table 2. MicroRNA Expression results in Behcet's patients and healthy controls

Accession	Gene	AVG ΔC_t		$2^{-\Delta C_t}$		Fold Change BD Group /Control Group	t-Test p value	Fold Up- or Down-Regulation BD Group /Control Group
		BD Group	Control Group	BD Group	Control Group			
MI0005532	miR-874	13.3	10.38	9.91E-05	0.000753	0.13	0.02757	-7.59
MI0003156	miR-518b	20.97	19.14	4.88E-07	1.73E-06	0.28	0.04595	-3.54
MI0000268	miR-34a	11.46	12.52	3.50E-04	1.70E-04	2.08	0.05476	2.08
MI0000285	miR-205	10.81	12.74	0.000556	0.000146	3.79	0.01482	3.79
MI0000780	miR-372	17.44	19.49	5.61E-06	1.36E-06	4.13	0.04818	4.13
MI0000299	miR-222	12.32	14.59	0.000195	4.05E-05	4.82	0.01115	4.82
	U6snRNA	14.47	16.75	4.40E-05	9.00E-06	4.89	0.04352	4.89
MI0000239	miR-197	16.09	18.46	1.43E-05	2.78E-06	5.16	0.03719	5.16
MI0000076	miR-20a	15.45	18.66	2.20E-05	2.40E-06	9.23	0.00133	9.23
MI0000773	miR-302c	12.08	15.45	0.000231	2.23E-05	10.34	0.04197	10.34
MI0000738	miR-302a	10.38	13.87	0.000748	6.66E-05	11.23	0.01963	11.23
MI0000747	miR-296	14.77	18.36	3.58E-05	2.98E-06	12	0.03453	12

Table 3. miRNAs associated diseases and pathways

MiRNA	Associated Disease	Related Pathway
mir-20a	B-Cell Lymphoma and Pulmonary Hypertension	Parkinsons Disease Pathway and DNA Damage Response
mir-34a	Melanoma and Retinoblastoma	MicroRNAs in cancer
U6snRNA	Poikiloderma With Neutropenia	mRNA Splicing - Major Pathway and Spliceosomal Splicing Cycle
mir-296	Esophagus Squamous Cell and Glioma	-
mir-205	Squamous Cell Carcinoma and Squamous Cell Carcinoma, Head And Neck	MicroRNAs in cancer
mir-302a	Teratocarcinoma and Embryonal Carcinoma	Preimplantation Embryo and Cell Differentiation
mir-302c	Non-Gestational Choriocarcinoma and Ovarian Germ Cell Cancer	Cell Differentiation and Mesodermal Commitment Pathway
mir-197	Thyroid Cancer, Nonmedullary, 2 and Diabetes Mellitus	Signaling by GPCR and Transmission across Chemical Synapses
mir-372	Testicular Germ Cell Tumor and Lung Cancer	DNA damage response and Mesodermal Commitment Pathway
mir-222	Tongue Squamous Cell Carcinoma and Thyroid Cancer, Nonmedullary	Cell Differentiation and miRNAs involved in DNA damage response
mir-518b	Psoriasis and Systemic Lupus Erythematosus	Psoriasis and Systemic Lupus Erythematosus
mir-874	Maxillary Sinus Cancer and Paranasal Sinus Cancer	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins

DISCUSSION

BD therapy also has numerous goals, which are to control medical symptoms, lessen inflammation, suppress the immune system, and prevent other organ damage. (20). Permanent and excessive cytokine production is a hallmark of the chronic inflammatory multisystem disease and may play a role in disorder pathogenesis. The serum ranges of TNF- α , interferon- γ , IL-1, 4, 6, 8, 10, 12, 13, 17, 18, 21, and IL-23 have been reported to be associated with BD (21-28). Therefore, cytokine neutralization is a beneficial therapeutic approach for the treatment of immune-mediated conditions (29). Although colchicine is one of the oldest recognized drugs, its mechanism of action isn't absolutely known. It is in all likelihood that colchicine will preserve to be a part of anti-inflammatory armament for a long time when used carefully and in an appropriate dosage (10). Colchicine is frequently used in BD for mucocutaneous involvement. The choice of treatment varies relying at the organs concerned and the severity of the disorder, the age and sex of the patient, and the period of the disease (30,31).

Altered MicroRNAs (miRNA) levels are observed in most inflammatory disorders and are considered to affect immunity through various mechanisms. Inflammatory responses have an effect on miRNA expression that regulates their biogenesis by changing the transcription and processing of leading transcripts or by affecting the stabilization of mature miRNAs. These circulating miRNAs may represent potential universal biomarkers for diagnosis and prognosis in inflammatory diseases such as Behcet's disease (17).

Recently, large-scale gene expression profiling has been conducted on the immune disease. In clinical studies, PBMC miRNAs haven't been studied as biomarkers for Behcet disease. Our results showed that up to 76 miRNAs were stably expressed PBMC and 12 different miRNAs were differently expressed in Behcet's patients.

Microarray and quantitative RT-PCR (qPCR) were the main approaches to analyze the expression profile of miRNA. Microarray can detect only known miRNAs according to the probe hybridization mechanism. qPCR has always been used as the gold standard way to measure the expression of miRNA, but only once could detect restricted kinds of miRNA, which has severely restricted its application. Recently, Dynamic Array Fluidigm technology has been developed. Fluidigm system was more concerned for its highest throughput per run. Therefore, the Biomark-Fluidigm machine changed into chosen to research the profile of miRNAs in Behcet's diseases. Different miRNA expressions were observed between BD and healthy control. miR-20a, miR-34a, miR-197, U6snRNA, miR-296, miR-205, miR-302a, miR-302c, miR-372 and miR-222 had been highly expressed and upregulated in BD. miR-518b was downregulated in miR-874 patients. It was previously reported that miR-20a, miR-34a, miR-197, miR-296, miR-302a also contribute to other chronic inflammatory diseases.

A typical example was miR-296, which turned into observed approximately twelve-fold up-regulated in Behcet patients compared with healthy control, and is also associated with atherosclerosis diseases. The expression level of miR-296 was found to be higher in atherosclerosis, which is considered an inflammatory disease regarding the vascular wall. The role of miR-296 on the regulation of angiogenesis, inflammatory response, cholesterol metabolism, and hypertension have been reported. Excessive expression of miR-296 can increase the expression level of VEGF, thereby inhibiting the Notch pathway. Also, miR-296 has been shown to increase the inflammatory response. The molecular targets of miR-296, which a position inside the development of inflammatory disease, can provide a basis for future research (32).

The position of miR-20 within the immune system has been recognized in a previous study. It has additionally been proven that overexpression of miR-20a inhibits TCR-mediated signaling but does not display the proliferation of primary human naïve CD4⁺T cells. However, miR-20a overexpression strongly suppresses IL-10 secretion and partially reduces the production of IL-2, 6, and IL-8, which are crucial regulators of inflammatory response. (33). Our result indicates that miR-20a overexpressed in Behcet patients.

Hou et al. were stated in this study the potential role of miR-34a-3p in the pathogenesis of Rheumatoid arthritis (RA). RA is a chronic inflammatory joint characterized by way of synovial inflammation. miR-34a-3p can be considered a promising therapeutic target for RA via inhibiting Fibroblast-like synoviocytes (FLS) proliferation and suppressing the manufacturing of pro-inflammatory cytokines and MMPs (34).

As a potential candidate, miR-302a can play an important role in macrophage cholesterol homeostasis. Mice treated with anti-miR-302a showed a more stable plaque morphology with fewer symptoms of inflammation, as well as reducing atherosclerotic plaque size by about 25% (35).

The changes miRNAs expression is mostly related to a specific pattern of cytokine expression; however, it appears that colchicine used during treatment does not increase miRNA expression associated with cytokine expressions (29). There are no cytokines in the target of miRNAs with increased expression. Only miR-222 levels in PBMCs have been discovered analogous to disease activity the increased production of pro-inflammatory cytokines. miR-221/222 expression was increased in patients with RA (36). Also, the expression level of miR-874 was found to be lower in BD patients than in the control group. Ahn et al. (2018) recognized the negatively regulated miR-874-5p and miR-30b-3p/PIK3CD and CX3CL1 pairs, which had been implicated in proteoglycan and cytokine-cytokine receptor interplay signaling pathways, suggesting them to be novel prognostic markers in Glioblastoma multiforme (GBM) which are exposed and activated by way of necrosis (37).

There are differences between studies investigating miRNA expressions in Behcet patients. For instance, in various studies conducted in Behcet patients, miR-146a, miR-155 expressions were found down or upregulated (15-18,38). In the study of Ibrahim et al, miRNA-146a expression was higher in Egyptian BD patients than the control group, and there was a significant relationship between miRNA-146a expression and BD's eye and vascular activity (15). In contrast, Pucetti et al. found downregulation of miRNA-146a expression in BD patients (17). Also, miR-155 expression was significantly decreased in PBMCs and DCs from BD patients with active uveitis and no differences were observed in controls (18). Contrary to this result, Na et al. show that miR-155 regulates Th17 immune response by targeting Ets-1. Suppression of miR-155 has been shown to reduce the number of pathogenic T cells that express IL-17. This situation was thought to be a potential therapeutic strategy for BD (16). There may be many reasons for different miRNA expression results. Conditions such as whether patients are in an active or quiescent period, whether patients under treatment or not, different drugs and dosages may create differences in miRNA expression results.

Jadideslam et al. investigated the potential role of miR-21, miR-146b and miR-326, it has been shown whether it will be used as a biomarker to predict diagnosis, organ involvement and to measure BD activity. This study showed down-regulated expression of miR-21 and up-regulated miR-326 in BD patients. Higher expression of miR-326 was observed in BD patients with uveitis and severe eye involvement. Sensitivity and specificity of these miRNAs were low for BD diagnosis. miR-326 expression rate measurement can be used as a biomarker for predicting uveitis and severe eye involvement in patients with BD (39). No difference was found in our study between the patient and control group.

In our study, miRNAs with altered expressions were compared with other studies with BD patients and no similar miRNA changes were observed. The detected miRNAs contribute clinically to the outcomes of colchicine at healing doses that have been less nicely established. Unfortunately, there are very few studies in the literature, there are no studies providing the same conditions. Table 4 provides a summary of similar studies.

Colchicine is utilized in other inflammatory diseases. Familial Mediterranean fever (FMF) is an inherited autoinflammatory disease that can lead to recurrent fever, serositis and skin rash attacks. The miRNA profile of patients dealt with colchicine had been examined and were found to be associated with inflammatory related gene expression levels of four miRNAs (miR-20a-5p, miR-197-3p, miR-574-3p, let-7d-3p) (40). miR-197 was located to be involved in lots of inflammatory pathways. miR-197 has been discovered to bind to the interleukin-1beta (IL-1 β) receptor, type I (IL1R1), one of the key molecules of inflammatory pathways. In FMF patients receiving daily colchicine treatment, miR-197 may contribute to understanding the inflammatory process (40).

In our study, the relationship of mirna detected with disease and pathways was observed and it was seen that this miRNAs were associated with cancer pathways, Table 3. Several studies have reported a relationship between Behcet's disease (BD) and various types of cancer. According to the meta-analysis consequences including 5 studies, especially, improved malignancy risk (pooled RR, 1.19; 95% CI: 1.09–1.30), particularly for hematological cancer (pooled RR, 2.58; 95% CI: 1.61–3.55) and thyroid cancer (pooled RR, 1.25; 95% CI: 1.04–1.47), a significant positive relationship was observed. However, high heterogeneity was also determined in the meta-analysis consequences ($I^2 = 81.3%$) (41). miR-20a, miR-34a, miR-197, U6snRNA, miR-205, miR-222, miR-296, miR-302a, miR-302c and, miR-372 were highly expressed and observed up-regulated in BD. miR-518b and miR-874 were observed downregulated in the patients. The miRNAs detected in this study and their associated diseases and pathways are summarized in Table 3.

miRNA expression alterations in these patients who use immunosuppressor or anti-inflammatory drugs and we think that this change will be important in the progression of the disease. Obviously, determining the healing efficacy and treatment target of colchicine can lead to the more effective management of these diseases.

CONCLUSION

In conclusion, we have defined 10 up-regulated miRNA and 2 down-regulated miRNA in BD patients using colchicine in the active period. These miRNAs are regarded to play a role in cancer-related diseases. In patients treated with colchicine, the change in the expression of these miRNA may give an idea of the impact on the progression of the disease. However, more studies are needed to fully understand its act and mechanism of impact in normal and pathological situations, therefore, miRNAs whose expressions are increasing and decreasing can potentially be used as a different view of BD treatment.

Conflict of interest : The authors declare that they have no competing interest.

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