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Title: Determination of mir-155 and mir-146a expression rates and its association with expression level of TNF-α and CTLA4 genes in patients with Behcet's disease

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Highlights

- MiR-155 and TNF-α expression was significantly increased, whilst CTLA-4 expression was significantly decreased in the PBMCs of BD patients.
- There was no significant difference in the miR-146a expression rate between BD patients and controls.
- A positive correlation between miR-155 and TNF-α expression and negative correlation between miR-155 and CTLA-4 expression were observed.
- No significant association was observed between the expression of miR-155, miR-146a, TNF-α and CTLA-4 genes with BD activity.
- MiR-155 and miR-146a expression rate were significantly higher in patients with uveitis and phlebitis, respectively.

Abstract

Introduction: MicroRNAs (miRNAs) are involved in the pathogenesis of inflammatory diseases. MiR-146 and miR-155 emerged as key regulators of the immune response. This study designed to analyze the miR-146a and miR-155 expression in patients with Behcet's disease (BD) and investigated their association with the expression of tumor necrosis factor-alpha (TNF-α) and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) genes.

Methodology: In a case-control study, 47 Iranian Azeri BD patients and 61 age- and sex matched healthy controls recruited to the study. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood tubes by Ficoll density-gradient centrifugation. Genomic DNA samples of BD and healthy controls were extracted using the rapid genomic DNA extraction

method from the peripheral blood collected in tubes containing EDTA. Total RNA was extracted from the PBMCs according to the TRIzol protocol. MiR-146a, miR-155, TNF-α and CTLA-4 expression were studied using real-time PCR.

Results: MiR-155 and TNF-α expression was significantly increased, whilst CTLA-4 expression was significantly decreased in the PBMCs of BD patients. There was no significant difference in the miR-146a expression rate between BD patients and controls. A positive correlation between miR-155 and TNF-α expression and negative correlation between miR-155 and CTLA-4 expression were observed. No significant association was observed between the expression of miR-155, miR-146a, TNF-α and CTLA-4 genes with BD activity. MiR-155 and miR-146a expression rate were significantly higher in patients with uveitis and phlebitis, respectively.

Discussion and conclusion

The expression of miR-155 increased in BD and associated with upregulation of TNF-α and downregulation of CTLA-4 genes.

Key words: Behcet's disease; miR-146a; miR-155; tumor necrosis factor-alpha (TNF-α) gene; cytotoxic T lymphocyte associated antigen-4 (CTLA-4) gene.

Introduction

Behcet's disease (BD) is a systemic vasculitis characterized by recurrent oral aphthous ulcers, genital ulcers, skin lesions and uveitis [1]. Although the etiology of BD has not been completely understood, the key pathophysiological event is the induction of an inflammatory response to environmental factors [2-4] in the presence of genetical susceptibility [2, 5] and epigenetic changes [6]. Innate and acquired immune system activation leads to production of proinflammatory cytokines including, interlukin-2 (IL-2), IL-6, IL-8, IL-12, IL-18, tumor necrosis factor-alpha (TNF-α), and interferon-γ [7]. TNF-α is secreted by Th1 lymphocytes and

macrophages and other antigen-presenting cells (APCs). TNF-α effects include activation of endothelial cells, macrophages and dendritic cells (DCs); stimulation of lymphocytes proliferation; and the synthesis of proinflammatory cytokines [7]. Increased circulating levels of TNF-α have been reported in BD [8]. Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is a member of the cell surface molecules, which expressed on activated and regulatory T cells, prevents co-stimulation by binding to CD80 and CD86 on APCs [9]. CTLA-4 plays a main role in inhibiting T cell activation and peripheral tolerance, in part, by inhibiting Th1 cytokine production [9]. Defective CTLA-4 expression and function are associated with autoimmune diseases [10]. However, previous association studies on the association between CTLA-4 and BD showed conflicting results [11-17].

MicroRNAs (miRNAs) are 19-23-nucleotide non-coding RNAs regulating gene expression through translational inhibition of target genes. Usually, miRNAs regulate biological functions by suppressing hundreds of target mRNAs inside a cell type [6]. Some miRNAs play a crucial role as fine regulators of the innate and adaptive immune system, and are involved in the pathogenesis of inflammatory diseases [18]. Among the various miRNAs playing a possible role in the control of the immune system, miR-146 (miR-146a and miR-146b) and miR-155 emerged as key regulators of the immune response. miR-146 is a multifunctional miRNA which increase apoptosis of DCs, suppress production of proinflammatory cytokines such as TNF-α, suppress Th1 and Th17 cells and activate T regulatory lymphocytes (Treg) [19]. MiR-146 deficiency leads to an excessive IL-6 and TNF-α production, myeloproliferative disorders and inflammatory disease [20]. MiR-155 activates DCs and increases inflammatory cytokines production; stimulates Th1 and Th17 cells differentiation and suppress CTLA-4 expression on activated T cells; stimulates Th2 immunity, B cells proliferation and antibody production; and required for

maturation and effector function of natural killer (NK) and CD8 cells [21, 22]. MiR-146α has been widely confirmed to be associated with immune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis [19, 23, 24]. Furthermore, aberrant expression of miR-155 has been reported in RA and SLE [25, 26]. Sonkoly et al. reported that miR-155 is overexpressed in patients with atopic dermatitis and increase the proliferative response of T cells by the downregulation of CTLA-4 [22]. Recently, differential expression of miRNAs in BD has been reported [27-31]. These studies suggest that miRNAs may provide clues to explain the different pathogenetic pathways leading to BD.

This study was designed to analyze the expression of miR-146a and miR-155 in patients with BD and investigated their association with the expression of TNF-α and CTLA-4 genes.

Methods & Materials

Subjects

In a case-control study, 47 Iranian Azeri BD patients diagnosed according the International Criteria for Behcet's Disease (IBCD) [32] and 61 age- and sex matched healthy controls recruited to the study. BD patients were recruited consecutively from the BD clinic of Connective Tissue Diseases Research Center between March 2016 and November 2017. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences and written informed consent was obtained from all participants.

Clinical and biochemical measurements

All patients were examined by a rheumatologist and disease activity was measured by Behcet's Disease Current Activity Form (BDCAF) [33] and Iranian Behcet's Disease Dynamic Activity

Measure (IBDDAM) [34, 35]. Eye disease activity was separately evaluated by Total Inflammatory Activity Index (TIAI) [34, 35].

Five mL of venous blood samples was collected after 12-h overnight fasting and peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood tubes by Ficoll (Lymphodex, Inno -Train, Germany) density-gradient centrifugation and immediately stored at -80 °C until use. Genomic DNA samples of BD and healthy controls were extracted using the rapid genomic DNA extraction (RGDE) method from the peripheral blood collected in tubes containing EDTA. Total RNA was extracted from the PBMCs according to the TRIzol protocol (Invitrogen, San Diego, CA), followed by reverse transcription using the reverse transcription reagent kit (Thermo Fisher scientific, USA). MiRNAs were isolated from 300 µL of plasma by reagent with MiRNeasy Biofluids isolation kit (Exiqon, Denmark) according to the manufacture's protocol and cDNA was synthesized using Universal cDNA synthesis kit (Exiqon, Denmark) by reverse transcription-polymerase chain reaction (RT-PCR) method. Then, purity and total RNA concentration were estimated by nanodrop ND1000 and purity of RNAs were assessed at 260-280 nm. The information of the TNF-α and CTLA-4 primers are shown in Table 1. Relative expression levels of miR-155, miR-146a, TNF-α and CTLA-4 were calculated using the ΔΔCt formula. The level of mRNA was normalized against that of beta-actin mRNA as internal reference gene for TNF-α and CTLA-4 genes and also based on the information contained within the Exiqon kit, mir-191 was used as a reference gene for miRNAs (mir-155, mir-146a) normalization. All tests were performed in at three biological repeats.

Statistical Analysis

Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., USA). Normal distribution of data was verified with the Kolmogorov–Smirnov test. Continuous variables were reported as means and standard deviations while categorical variables were expressed as frequency and percentage. One sample T test was used for comparing genes expression with constant value. Comparisons between groups were made by chi-squared test and independent sample *t* test as appropriate. Analysis of covariance (ANCOVA) was used to identify any differences between the 2 groups, adjusting for covariates. P-value less than 0.05 was considered as statistically significant.

Results

In a case control study, expression of miR-146a, miR-155 and TNF-α and CTLA-4 genes were screened in PBMCs of 47 patients with BD and 61 healthy controls. Demographic characteristics of studied groups are shown in Table 2. Detailed clinical and laboratory findings of the enrolled BD patients are presented in Table 2. The results showed that the miRNA-155 and TNF-α expression was significantly increased, whilst CTLA-4 expression was significantly decreased in the PBMCs of BD patients compared to healthy controls (Fig 1). Furthermore, there was no significant difference in the miR-146a expression rate between BD patients and controls (Fig 1). A positive correlation between miR-155 and TNF-α expression and negative correlation between miR-155 and CTLA-4 expression was observed (Table 3). Correlation between miR-146a and TNF-α and CTLA-4 expression was non-significant (Table 3).

We analyzed correlation between the expression rate of miR-146a, miR-155, TNF-α and CTLA-4 genes and clinical characteristics of BD patients. MiR-155 expression rate was significantly higher in BD patients with uveitis (Table 4). Furthermore, expression rate of miR-155 in patients

with vision loss was more than other patients, although it did not rich to significant level. MiR-146α expression rate was significantly higher in patients with phlebitis (Table 4). We also investigated correlation between the expression of miR-155, miR-146α, TNF-α and CTLA-4 genes with BD activity. No significant association was observed (Table 5).

Discussion

MiRNAs have been shown to play a critical role in the immune system function and inflammatory response by regulating the differentiation of various immune cell subsets [36-38]. In the present study, two known immunologically relevant miRNAs based on miRBase and relevant reports were selected as candidates and their expression was investigated in the BD patients and healthy controls. The results showed that expression of miR-155 significantly upregulated in the Azeri patients with BD. There was no significant difference in the expression of miR-146a in studied groups. We also investigated the expression rate of TNF-α and CTLA-4 genes, which these miRNAs have important role in their regulation. We observed that expression of TNF-α gene significantly upregulated and expression of CTLA-4 significantly downregulated in the Azeri patients with BD. Correlation between miR-155 expression with TNF-α and CTLA-4 genes expression was detected. Correlation between miR-146α expression with TNF-α and CTLA-4 genes expression was non-significant. Significant association was observed between miR-155 expression with uveitis and miR-146a expression with phlebitis.

Contrary to the result of this study, Zhou et al. reported a decreased miR-155 expression in PBMCs of BD patients with active uveitis compared to BD patients with inactive uveitis and healthy control [28]. Differences between BD patients with inactive uveitis and healthy control was not significant. They showed that miR-155 inhibits the production of IL-6 and IL-1b and

increases the production of IL-10 by immature DCs. On the contrary, NA et al. reported that expression of miR-155 significantly increased in CD4+ T cells of patients with active BD [39]. They showed that repression of miR-155 in CD4+ T cells increased Ets-1 expression and reduced the number of IL-17 expressing T cells and overall IL-17 production. They showed that miR-155 suppression in CD4 + T cells increased the expression of Ets-1 and reduced the number of T cells expressing IL-17 and production of IL-17.

Our results about miR-146a expression rate was somewhat different from previous studies indicating an association between miR-146a with immune diseases such as RA, SLE and psoriasis [19-21]. Lu et al. reported that miR-146a is important for Treg cells inhibition and miR-146a deficiency in Treg cells led to breakdown of immunological tolerance [40]. Furthermore, Tang et al. indicated decreased miR-146a expression rate in SLE patients [41]. Zhou et al. in a study on 809 patients with BD in a Chinese population showed decreased frequency of the homozygous rs2910164 CC genotype and C allele in BD patients compared with controls. MiR-146a expression rate in PBMC and IL-7, TNF-α, and IL-1β production in CC cases was significantly lower than GG cases (42). In another study by Ibrahim et al. expression of miR-146a in Egyptian patients with BD was significantly higher than controls [43]. They could not find any association between miR-146a expression level and organ involvement in BD patients. However, a significant association was observed between miR-146a expression and eye and vascular disease activity. On the contrary, Hou et al. in their study on 377 patients with BD and 660 healthy control reported that miR-146a did not confer susceptibility to BD [44].

miRNAs are oligonucleotide RNAs that are not only detectable in tissue cells but also detectable in extracellular fluids (ECF) such as plasma, urine and others [45]. They are remarkably stable in ECF, so they can be detected and measured [45]. Therefore, miRNAs can be used as an

accessible disease biomarker. miRNAs measurement has been suggested for early diagnosis, staging, follow up, assessment of therapeutic responses and therapy outcomes in malignancies [45]. Downreulation and upregulation of miRNAs can change immune response, so they can be used as therapeutic target in inflammatory diseases [45]. Although, our study did not show the association between BD activity with miR-155 and miR-146α expression rates, the increase in the expression of miR-155 and its association with upregulation of TNF-α and downregulation of CTLA-4 genes was observed. Therefore, it may be used as a diagnosis biomarker and therapeutic target for BD in the future.

Present study had some limitations. a) Sample size was small. b) Although our results showed a correlation between increased expression of miR-155 and upregulation of TNF-α and down regulation of CTLA-4 genes in PBMC of BD patients, establishing a causal relationship between them is not reliable. The proof requires an invitro study in which the effect of repressing miR-155 on the expression of TNF-α and CTLA-4 genes was investigated.

The expression of miR-155 has increased in BD and associated with upregulation of TNF-α and downregulation of CTLA-4 genes. MiR-155 over-expression is associated with uveitis. There was no significant difference in the miR-146a expression rate between BD patients and controls.

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Table 1. PCR primers and product size

Target	Primer	Target size
TNF-α	FM-TNF: GCAGGTCTACTTTGGGATCATT RM-TNF: AGAAGAGGTTGAGGGTGTCT	117
CTLA4	Forward:GTCACTCGATGTCATTCC Reverse: GCCTCTAGCCCAAATTGT	150

Table 2. Demographic, clinical and laboratory characteristics of participants

	Behcet's disease group (N=47)	Healthy control group (N=61)	P-value
Age (mean ± SD) years	38.1 ± 10.3	37.4 ± 8.5	NS
Gender (male:female)	29/18 (1.6)	37/24 (1.5)	NS
Oral aphthous ulcer (%)	45 (95.7)	-	-
Uveitis (%)	34 (72.3)	-	-

Genital ulcer (%)	23 (48.9)	-	-
Positive Pathergy test (%)	19 (40.4)	-	-
Pseudofolliculitis (%)	11 (23.4)	-	-
Arthritis (%)	9 (19.1)	-	-
Erythema nodosum (%)	8 (17)	-	-
Phlebitis (%)	5 (10.6)	-	-
Central nervous system involvement	1 (2.1)	-	-
HLA-B5 (%)	27 (57.4)	-	-
HLA-B51 (%)	25 (53.2)	-	-

SD: standard deviation; NS: non-significant; NS: non-significant

Table 3. Association between miR-146a and miR-155 with TNF-α and CTLA-4 genes expression in patients with BD

	TNF-α		CTLA-4	
	Pearson correlation	P-value	Pearson correlation	P-value

Mir-146a	-0.049	0.617	0.030	0.756
Mir-155	0.391	0.0001	-0.274	0.004

TNF-α: tumor necrosis factor-α; CTLA-4: cytotoxic T-lymphocyte associated protein-4

Table 4. Correlation between demographic and clinical characteristics with miR146a, miR-155, TNF-α and CTLA-4 expression rate.

Demographic and clinical features	miR-146a expression rate (mean±SD	P-value	miR-155 expression rate (mean±SD)	P-value	TNF-α expression rate (mean±SD)	P-value	CTLA-4 expression rate (mean±SD)	P-value
Sex								
Male	1.42±0.4	0.868	2.98±1.7	0.767	2.58±0.9	0.121	2.15±0.8	0.534
female	1.41±0.2		3.03±1.7		2.28±0.9		2.06±0.7	

Genital ulcer								
Positive	1.56±0.5	0.017	4.21±1.8	0.941	2.99±0.8	0.265	1.63±0.4	0.145
Negative	1.29±0.2		4.27±1.5		3.21±0.88		1.88±0.6	
Skin lesions								
Positive	1.47±0.5	0.615	4.28±1.7	0.918	3.15±0.7	0.766	1.82±0.7	0.423
Negative	1.41±0.3		4.22±1.5		3.07±0.9		1.69±0.4	
MSK involvement								
Positive	1.41±0.3	0.901	3.49±1.8	0.144	3.31±0.9	0.443	1.96±0.8	0.225
Negative	1.44±0.4		4.41±1.5		3.06±0.8		1.71±0.5	
Pathergy								
Positive	1.34±0.2	0.411	4.39±1.7	0.838	3.21±0.9	0.704	2.21±0.9	**0.012**
Negative	1.48±0.5		4.25±1.6		3.08±0.7		1.64±0.3	
Uveitis								
Positive	1.45±0.4	0.591	4.70±1.4	**0.001**	3.14±0.8	0.778	1.80±0.6	0.214
Negative	1.37±0.2		2.86±1.3		3.07±0.7		1.55±0.2	
Vision loss								
Positive	1.36±0.4	0.652	5.57±0.6	0.056	3.22±0.9	0.824	1.63±0.2	0.619
Negative	1.44±0.3		4.14±1.6		3.13±0.8		1.76±0.6	
Phlebitis								
Positive	1.87±0.9	**0.006**	4.49±2.1	0.719	3.14±0.5	0.909	1.71±0.3	0.901
Negative	1.38±0.2		4.22±1.5		3.09±0.8		1.74±0.6	

As shown in the table, items that have a statistically significant difference are shown as **Bold**.

TNF-α: tumor necrosis factor- α; CTLA-4: cytotoxic T-lymphocyte associated protein-4; SD: standard deviation.

Table 5. Correlation between miR-146a, miR-155, TNF-α and CTLA-4 genes expression and Behcet's disease activity

	Patients age at the disease presentation		BDCAF		IBDDAM		TIAI	
	Pearson correlation	P-value	Pearson correlation	P-value	Pearson correlation	P-value	Pearson correlation	P-value
Mir-146a	0.082	0.590	- 0.138	0.414	0.014	0.937	- 0.085	0.617
Mir-155	- 0.096	0.524	0.021	0.900	0.121	0.475	0.237	0.158
TNF	0.008	0.590	0.010	0.995	- 0.144	0.394	- 0.061	0.718

CTLA-4	0.016	0.917	0.015	0.930	- 0.050	0.770	0.300	0.071

TNF-α: tumor necrosis factor-α; CTLA-4: cytotoxic T-lymphocyte associated protein-4; BDCAF: Disease Current Activity Form; IBDDAM: Iranian Behcet's Disease Dynamic Activity Measure

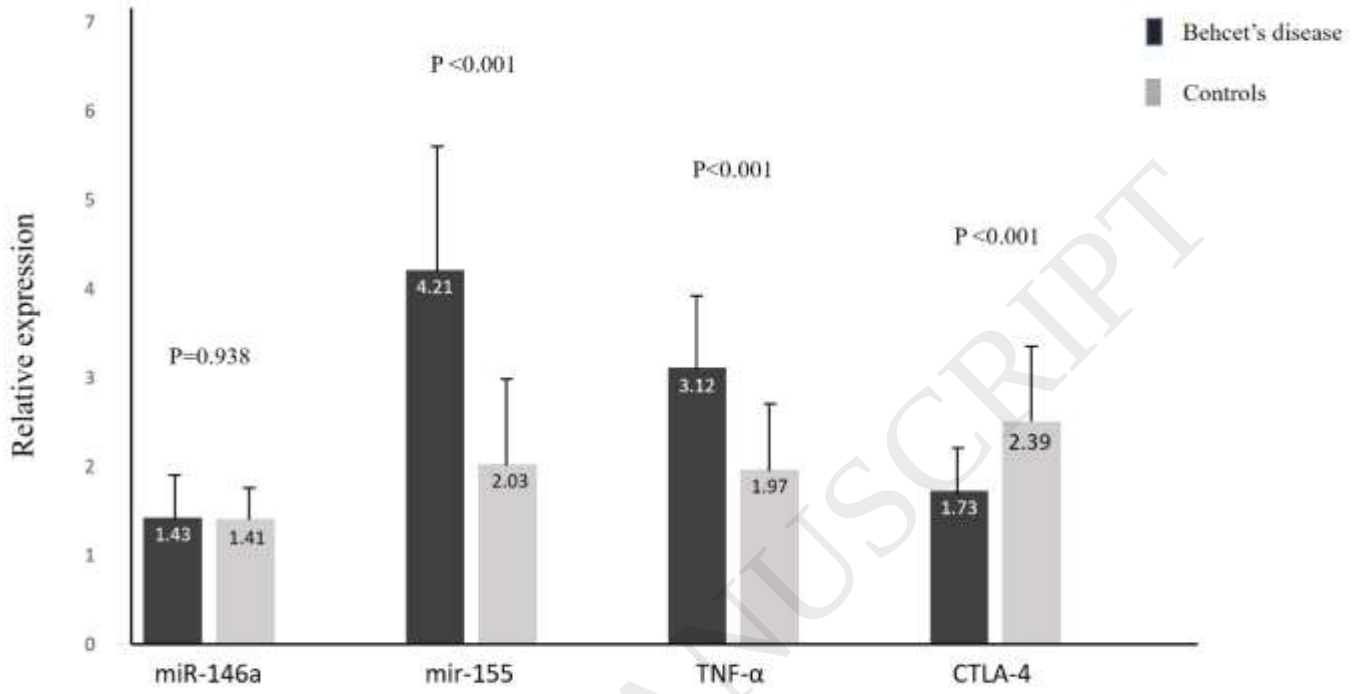

Fig 1. Relative expression of miR-146a, miR155, TNF-α and CTLA-4 in studied groups.