Research Article

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Biomarkers in Medicine

Expression levels of miR-21, miR-146b and miR-326 as potential biomarkers in Behcet's disease

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Aim: Behcet's disease (BD) is a vasculitis. Lines of evidence suggest miRNAs as diagnostic and prognostic markers in autoimmune diseases. This study was designed to investigate the potential role of miR-21, miR-146b and miR-326 as biomarkers for diagnosis, predicting organs involvement and measuring BD activity. **Patients & methods:** In this cross-sectional study, the study groups consisted of 46 BD patients and 70 age- and sex-matched healthy volunteers. The expression rates of three miRNAs were determined by quantitative real-time PCR. **Results:** Our results demonstrated significantly lower expression of miR-21 and miR-146b and higher expression of miR-326 in BD patients. MiR-21 expression rate in patients with severe eye involvement and miR-326 expression rate in patients with uveitis and severe eye involvement were increased. **Conclusion:** MiR-326 expression rate can be used as a biomarker for prediction of uveitis and severe eye involvement in patients with BD.

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Keywords: Behcet's disease • biomarker • miR-146b • miR-21 • miR-326 • miRNA • uveitis

Behcet's disease (BD) is a complex disease characterized by recurrent oral aphthous ulcers, genital ulcers, skin lesion and uveitis. Environmental factors, immune system dysregulations, genetic and epigenetic factors role have been introduced in the pathogenesis of BD. Epigenetic is outcome of genetic and environmental factors that modifies gene expression pattern by internal and external interfering elements [1–5]. MiRNAs are small noncoding RNAs that conduct gene expression at the post-transcriptional level through sponging of target mRNAs [1]. MiRNAs serve as key controllers of various processes as in early development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction and organ development. They also play central role in the normal function of the immune system, tolerance and autoimmunity. Individual miRNAs modulate inflammation and other immune responses during infections, cancer and autoimmune diseases [6–8]. There are many uncertainties about the role of miRNAs in autoimmune disorders. The advents of small noncoding RNA sequencing technologies enable researchers to study expression profiles of many miRNAs. Furthermore, antisense and precursor available technologies are new methods to study the influence of differential miRNA expression *in vivo* and *in vitro*. A number of studies have been suggested that individual miRNA may mildly regulate the expression of great numbers of genes, but this mild regulation of individual genes results in notable results on whole signaling pathways and a powerful biological outcome [1].

The role of many cytokines such as interferons (IFN), TNF-α, IL-2, IL-6, Il-10, IL-11, IL-12, IL-18, IL-19, IL-20, IL-26, IL-27, IL-28, IL-29, IL-32, IL-34, IL-35 has been introduced in the pathogenesis of BD [9–12]. In

addition, the association between cytokines and the involvement of various organs in BD has also been reported. For example, it has been shown that IFN-γ is involved in the inflammatory process during BD uveitis; whereas IL-10 looks to have a protective characteristic [13]. It has been shown that epigenetic factors play an important role in regulating cytokines in patients with BD [14–17].

Lines of evidence suggest miRNAs as diagnostic and prognostic markers in autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis, Type 1 and 2 diabetes mellitus, systemic lupus erythematosus (SLE), Sjogren's syndrome, psoriasis, Familial Mediterranean fever, primary biliary cirrhosis, inflammatory bowel diseases, autoimmune hepatitis and idiopathic thrombocytopenic purpura (ITP) [1,18,19]. MiR-21 has an anti-inflammatory effect on injury site macrophages, and it may play an important role in resolution of the inflammation beside repressing effect on IL-12 [15,20]. Overexpression of miR-21 provides unlimited cell growth with inhibited apoptosis to provoke development of chronic inflammatory diseases [21]. MiR-326 is participated in proliferation, maturation, differentiation (precisely Th17) and maintenance of immune homeostasis. MiR-326 overexpression is reported to be directly or indirectly correlated with the pathogenesis of autoimmune diseases including SLE, ITP, MS and Type 1 diabetes mellitus. Also, miR-326 silencing in experimental autoimmune encephalomyelitis is reported to decrease the number of Th17 cells and severity of disease [1]. V-ets avian erythroblastosis virus E26 oncogene homolog 1 (*Ets-1*) participates in differentiation of Th17, hematopoietic development, angiogenesis and tumor progression, which is introduced as appealing target to sponge by miR-326 [1]. MiR-146 family targets IRAK1 and tTRAF6. IRAK1 and TRAF6 are modulatory factors in TLR signaling that is dysregulated in autoimmune diseases [22]. Recently, studies have been conducted to use miR-146a and miR-21 as a target for treatment of autoimmunity diseases in the preclinical phases [23].

Because of the lack of specific laboratory findings, BD diagnosis is based on clinical criteria; therefore, finding of diagnostic biomarkers can be helpful for diagnosis of BD. In the present study, potential role of miR-21, miR-146b, miR-326 was assessed as diagnostic biomarkers in BD. Therefore, we designed a cross-sectional study on Iranian Azari patients with BD to investigate the potential role of miR-21, miR-146b and miR-326 as biomarkers for diagnosis, predicting organs involvement and measuring BD activity.

Patients & methods

Study groups

The study groups consisted of 46 BD Iranian Azari patients and 70 age-, sex- and ethnically-matched healthy volunteers (Table 1). The diagnosis of BD was conducted by the International Criteria for Behcet's Disease [24]. BD patients were enrolled to the study consecutively from a BD registry in the Connective Tissue Diseases Research Center. All BD patients except three (93.5%) had active disease, were not relative and were treated with various medications (Table 1). All subjects in this study provided informed consent, and the study had been approved by the Ethics Committee of the Tabriz University of Medical Sciences (TBZMED.REC.1395.1357). BD activity was measured by Behcet's Disease Current Activity Form, Iranian Behcet's Disease Dynamic Activity Measure and Total Inflammatory Activity Index (TIAI) [25–27]. Behcet's Disease Current Activity Form measures BD activity in all organs, but Iranian Behcet's Disease Dynamic Activity Measure measures BD activity in all organs except the eye and TIAI measures BD activity in the eye [25,26]. Patients with retinal vacuities and/or posterior uveitis considered as severe eye involvement. Collected demographic data for patients are summarized in Table 1.

RNA extraction & cDNA synthesis

5 ml of peripheral blood was obtained from participants. Peripheral blood mononuclear cells isolated by Ficoll (Lymphodex, Inno-Train, Kronberg im Taunus, Germany) density-gradient centrifugation (Sigma- Aldrich GmbH, Munich, Germany) according to manufacture instruction. Subsequently total RNA extraction performed by TRIzol® (Invitrogen, CA, USA) based on the manufacturer's protocol and quality of the product checked using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, DE, USA). Extracted RNA was stored at -80°C until complementary DNA (cDNA) synthesis (Thermo Fisher Scientific, MA, USA).

Quantitative PCR

The miRNAs expression was evaluated by minimum inhibitory concentration (MIC) real-time instrument (BioMolecular Systems, Sydney, Australia). Quantitative reverse transcription PCR (qRT-PCR) was provided employing ExiLENT SYBER Green Master mix (Exiqon, MA, US) against LNA based primer sets (Exiqon). The miRNA primers were purchased from Exicon Company (Woburn, MA, USA). q-PCR was performed with 4 µl

Table 1. Demographic, clinical and laboratory characteristics of participants and medications.

Clinical parameters	Behcet's disease group (n = 46)	Healthy control group (n = 70)	p-value
Age, years (mean ± SD)	39.82 ± 11.1	37.08 ± 7.6	0.091
Gender (male/female)	29/17 (1.71)	46/24 (1.92)	0.769
Oral aphthous ulcer	44 (95.7)		
Uveitis	33 (71.7)		
Genital ulcer	23 (50)		
Positive pathergy	14 (30.4)		
Pseudofolliculitis	11 (23.9)		
Arthritis	9 (19.6)		
Erythema nodosum	9 (19.6)		
Phlebitis	6 (13)		
Epididymitis	3 (6.5)		
CNS involvement	1 (2.2)		
HLA-B5	27 (58.7)		
HLA-B51	25 (54.3)		
Medications:			
– Colchicine	24 (52.2)		
– Prednisolone	22 (47.8)		
– Azathioprine	22 (47.8)		
– Methotrexate	16 (34.8)		
– NSAIDs	12 (26.1)		
– IFN-α	3 (6.5)		
– Cyclophosphamide	2 (4.3)		
– Cyclosporine	2 (4.3)		
– Sulfasalazine	1 (2.2)		

Data shown as n (%) unless otherwise stated.
CNS: Central nervous system; NSAID: Nonsteroidal anti-inflammatory drug.

of diluted DNA, 1 µl miRNA primer and 5 µl of master mix followed by initial enzyme activation at 95°C for 10 min; 45 cycles of denaturation for 10 s at 95°C; annealing on 60 s at 60°C. MiR-21, miR-146b and miR-326 genes' expression were normalized to miR-191 as internal reference.

Statistical analysis

The results analyzed by SPSS software version 25.0 (SPSS, IL, USA). Normal distributions experimented by the Kolmogorov–Smirnov test with Lilliefors correction. Quantitative data were showed by the mean ± standard deviation, while categorical variables were expressed as frequency and percentage. Expression of miR-21, miR-146b and miR-326 between groups was analyzed by Mann–Whitney U test. Pearson correlation analysis was adopted to evaluate the correlation between study variables with clinical characteristics. The receiver-operating characteristic (ROC) curve analysis was performed to identify the optimal cutoff points of miR-21, miR-146b and miR-326 expression for predicting BD and clinical manifestations of BD. The area under the curve (AUC) was calculated to evaluate the diagnostic performance of these miRNAs and $p < 0.05$ was considered statistically significant.

Results

In this cross-sectional study, miR-21, miR-146b and miR-326 expression were compared between BD and control groups. Demographic, clinical and laboratory characteristics of studied groups were showed in Table 1.

Our results demonstrated underexpression of miR-21 and overexpression of miR-326 in BD patients (0.97 ± 0.71, 1.40 ± 0.82, respectively) compared with controls (1.54 ± 1.16 and 1.13 ± 0.93, respectively; Figure 1). However, no significant difference was observed for miR-146b expression in BD and control groups (1.17 ± 0.72 and 1.38 ± 0.91, respectively; Figure 1).

In addition, we analyzed associations between miR-21, miR-146b and miR-326 expression rates and clinical characteristics of BD patients (Table 2). Our results showed significantly higher miR-21 expression rate in patients

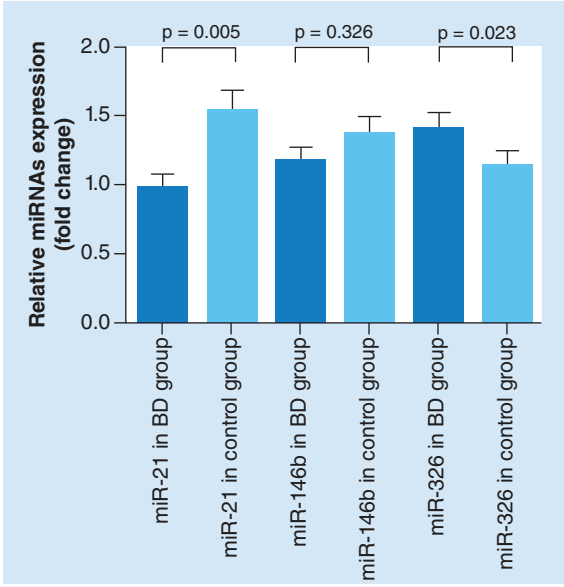

Figure 1. Expression of miR-21, miR-146b and miR-326 in Behcet's disease patient compared with healthy controls.
BD: Bechet's disease.

Table 2. Clinical profile of patients with miRNAs expression.

Demographic and clinical characteristics	Frequency, n (%)	Change fold of miR-21 expression (mean ± SD)	p-value	Change fold of miR-146b expression (mean ± SD)	p-value	Change fold of miR-326 expression (mean ± SD)	p-value
Gender							
Male	29 (63)	0.95 ± 0.79	0.663	1.04 ± 0.66	0.259	1.39 ± 0.87	0.938
Female	17 (37)	1.01 ± 0.57		1.39 ± 0.78		1.42 ± 0.75	
Genital ulcer							
Yes	23 (50)	1.00 ± 0.75	0.784	1.10 ± 0.72	0.423	1.30 ± 0.79	0.613
No	23 (50)	0.94 ± 0.39		1.23 ± 0.73		1.51 ± 0.85	
Uveitis							
Yes	35 (76.1)	1.08 ± 0.71	0.124	1.21 ± 0.66	0.396	1.66 ± 0.79	<0.001*
No	11 (23.9)	0.65 ± 0.28		1.08 ± 0.80		0.65 ± 0.27	
Severe eye involvement							
Yes	25 (54.3)	1.18 ± 0.75	0.030*	1.17 ± 0.64	0.465	1.92 ± 0.69	<0.001*
No	21 (45.7)	0.72 ± 0.39		1.15 ± 0.83		0.70 ± 0.22	
Positive pathergy							
Yes	14 (30.4)	0.67 ± 0.51	0.004*	0.94 ± 0.62	0.401	1.31 ± 0.83	0.101
No	32 (69.6)	1.37 ± 0.62		1.13 ± 0.69		1.67 ± 0.78	
Pseudofolliculitis							
Yes	11 (23.9)	0.93 ± 0.83	0.722	1.22 ± 0.76	1.000	1.44 ± 0.88	0.899
No	35 (76.1)	0.98 ± 0.38		1.16 ± 0.72		1.39 ± 0.81	
Erythema nodosum							
Yes	9 (19.6)	0.77 ± 0.53	0.445	0.77 ± 0.44	0.031*	0.76 ± 0.29	0.007*
No	37 (80.4)	1.02 ± 0.45		1.27 ± 0.74		1.56 ± 0.83	
Arthritis							
Yes	9 (7.8)	0.92 ± 0.73	0.978	0.91 ± 0.59	0.338	1.16 ± 0.80	0.386
No	37 (31.9)	0.98 ± 0.32		1.23 ± 0.74		1.46 ± 0.82	
Phlebitis							
Yes	6 (13)	0.89 ± 0.73	0.886	1.60 ± 0.93	0.241	1.17 ± 0.75	0.473
No	40 (87)	0.98 ± 0.32		1.10 ± 0.67		1.44 ± 0.83	

*Significant values.
SD: Standard deviation.

Table 3. Correlation between the miRNAs expression and rates of Behcet's disease activity.

miRNAs	BDCAF		IBDDAM		TIAI	
	Pearson correlation	p-value	Pearson correlation	p-value	Pearson correlation	p-value
MiR-21	0.009	0.954	-0.163	0.289	0.331	0.025*
MiR-146b	-0.230	0.143	0.007	0.964	-0.014	0.925
MiR-326	0.137	0.385	0.094	0.544	0.554	<0.001*

*Signidicant values.
BDCAF: Behcet's disease current activity form; IBDDAM: Iranian Behcet's disease activation form; TIAI; Total inflammatory activity index.

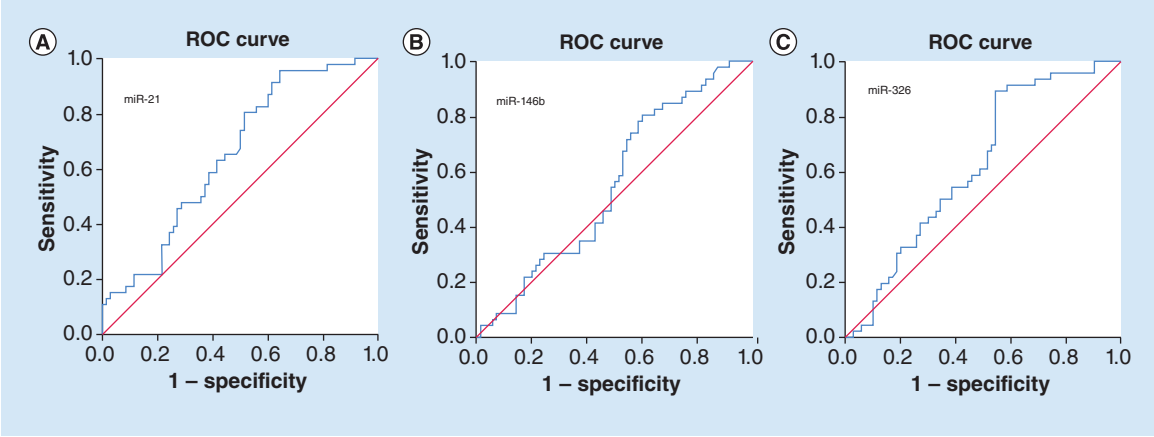

Figure 2. Receiver-operating characteristic curve analysis of the diagnostic value of miRNAs in Behcet's disease. ROC curve analysis showed that these miRNAs may serve potential targets for monitoring, diagnosing and/or treating Behcet's disease. The area under the curve, sensitivity and specificity serve the accuracy in prediction.
ROC: Receiver-operating characteristic.

Table 4. Results of receiver-operating characteristic curve analysis.

miRNA	AUC	Asymptotic 95% CI	Sensitivity	Specificity	p-value
miR-21	0.653	(0.554–0.752)	82.6	44.3	0.005*
miR-146b	0.554	(0.449–0.659)	78.3	41.4	0.325
miR-326	0.625	(0.524–0.726)	89.1	45.7	0.023*

*Significant values.
AUC: Area under the curve.

with severe eye involvement and higher miR-326 expression rate in patients with uveitis and severe eye involvement. MiR-21 expression in patients with positive pathergy and miR-146b and miR-326 expression in patients with erythema nodosum (EN) were significantly lower than patients without these symptoms (Table 2).

The Pearson correlation analysis was utilized to analyze the correlation between miRNAs with BD activity and results are summarized in Table 3. A positive and significant correlation was observed between miR-21 and miR-326 expression and TIAI.

In order to assess the potential of miR-21, miR-146b and miR-326 for diagnosis of BD, ROC curve analysis was performed and the AUC was calculated by calculating sensitivity and specificity for each possible cutoff point of the miR-21, miR-146b and miR-326 expression levels (Figure 2). Our results showed a relatively high sensitivity of miR-21 and miR-326 for diagnosis of BD (Table 4). However, the specificity of them was very low (Table 4).

In addition, we performed ROC curve analysis for assessing the ability of miRNAs to predict clinical manifestations of BD (Figure 3). MiR-326 had a moderate sensitivity and high specificity for prediction of uveitis in patients with BD (Table 5). Sensitivity and specificity of miR-326 for prediction of severe eye involvement was high (Table 5).

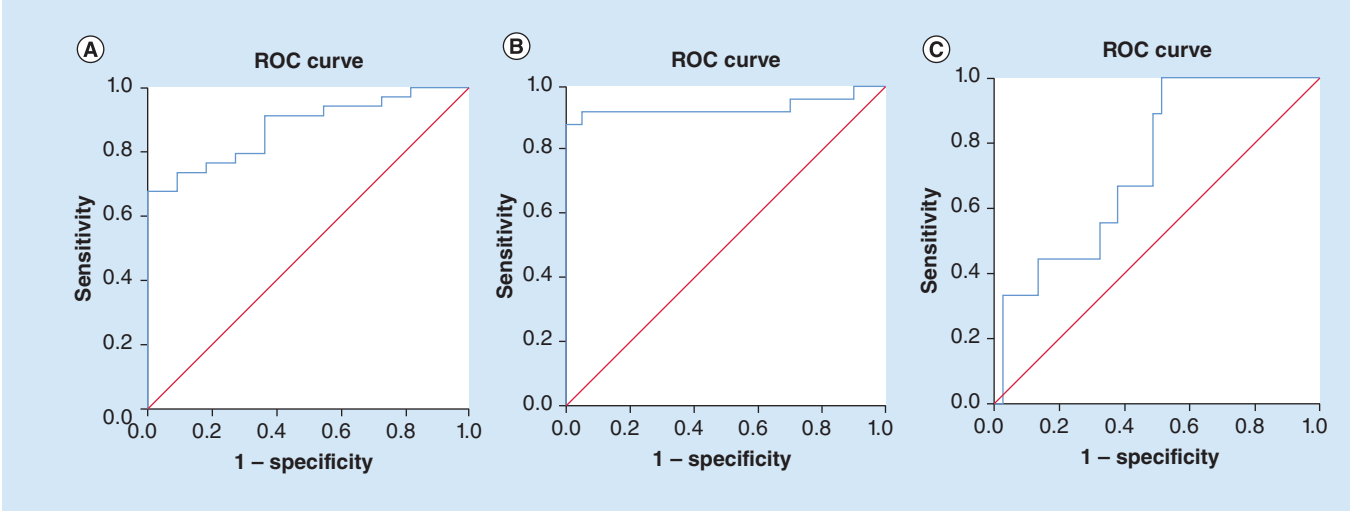

Figure 3. Receiver-operating characteristic curve analysis of miRNAs with clinical characteristics. ROC curves show the specificity and sensitivity levels and 95% CI between miR-326 white ocular **(A)**, severe eye involvement **(B)** and miR-146b with erythema nodosum **(C)** in Behcet's disease patients.
ROC: Receiver-operating characteristic.

Table 5. Results receiver-operating characteristic curve analysis of miRNAs with clinical characteristics.

MiRNA and clinical manifestations of BD	AUC	Asymptotic 95% CI	Sensitivity	Specificity	p-value
MiR-326, uveitis	0.877	(0.777–0.977)	73.5	90.9	0.001*
MiR-326, severe eye involvement	0.934	(0.847–1.000)	92	90	0.001*
MiR-146b, erythema nodosum	0.733	(0.569–0.897)	88.9	51.4	0.032*

*Significant values.
BD: Behcet's disease; AUC: Area under the curve.

Discussion

The present study showed lower expression of miR-21 and higher expression of miR-326 in patients with BD compared with healthy controls. We found higher expression of miR-326 in BD patients with uveitis and severe eye involvement and higher expression of miR-21 in BD patients with severe eye involvement. Although we could not find a high sensitivity and specificity of these miRNAs for diagnosis of BD, we found 73.5% sensitivity and 90.9% specificity of miR-326 to predict uveitis in patients with BD. The sensitivity and specificity of miR-326 for prediction of severe eye involvement were 92 and 90%, respectively. We could not find any correlation between miR-21 and miR-146b expression rates and BD activity. However, a positive and significant correlation was observed between miR-326 expression rate with TIAI.

Nonetheless, recent studies focus on epigenetic mechanisms that regulate gene expression without alteration of DNA sequence [28]. There is a lot of uncertainty about the role of miRNAs in the pathogenesis of various diseases, especially autoimmune/autoinflammatory disorders. The advents of small noncoding RNA sequencing technologies enable researchers to study expression profiles of many miRNAs. Furthermore, antisense and precursor available technologies are new methods to study the influence of differential miRNA expression *in vivo* and *in vitro*. A number of studies have been suggested that individual miRNAs can have a small effect on the expression of a large number of genes. However, the sum of these small effects causes significant changes in signaling pathways and a powerful biological outcome [1]. Many investigators were introduced miRNAs as interesting biomarkers in pathology and novel approaches were improved for the efficient transferring of miRNA mimicries, pre-miRNAs or antisense oligos for treatment of cancers and some technologies even reached to clinical trial stage [28].

Exact experimental methods will help us in understanding how and why these miRNAs interfere with health and disease and the resulting information can be used to identify new treatments for diseases. Recently, researches

about miRNAs in BD have begun, but there is still a long way to fully understand their role in BD pathogenesis. Ibrahim *et al.* in a study in the Egyptian population showed miR-146a polymorphism in BD patients [29]. They showed a significant association between miR-146a expression and the central nervous system, eye and vascular involvement in BD [29]. Another study has shown that the expression of miR-638, miR-4488 and miR-3591-3p is associated with an increase in the expression of IL-6 in BD [30].

The expression pattern of miR-21, miR-146b, miR-326 and their potential target transcripts can affect the differentiation of $CD4^+$ T cells toward Th1, Th2 or Th17 responses [1,31]. Recently, a study has shown that silencing miR-21 produces a significant resistance to experimental autoimmune encephalomyelitis [18]. Critical role of miR-21 has been confirmed in pathogenesis of autoimmune diseases such as Type 1 and Type 2 diabetes mellitus, SLE, psoriasis, inflammatory bowel diseases and ITP, as well as malignancies such as colorectal cancer, non-small-cell lung cancer, lung cancer, brain cancer, glioma, breast cancer, ovarian cancer and hepatocellular carcinoma [1,6,15,18,32–34]. Recently, reported that one of the miR-21 targets is IL-10, an anti-inflammatory cytokine. MiR-21 expression is especially decreased in IL-10-producing B-cell regulatory cell, resulting in decreased production of IL-10 in patients with autoimmune disorders. This miRNA can be a potential therapeutic target for the improvement of autoimmune therapies. Inhibition of miR-21 expression *in vivo* may be a useful therapeutic strategy for the treatment of autoimmune diseases [35].

MiR-326 has critical role in the regulation of Th17 differentiation and hence contributes to the pathogenesis of BD [1]. MiR-326 produces IL-17 by increasing the differentiation of Th17 cells [1]. MiR-326 mediates Th17 differentiation by interference with the translation of Ets-1, which is a negative regulator of Th17 differentiation [1]. Recent studies have shown that changes in the expression of miR-326 in Th17 contribute to the pathogenesis of MS and are associated with the severity of the disease [1]. The role of miR-326 has been shown in the pathogenesis of Type I diabetes mellitus, SLE and ITP [1]. With the introduction of Th17 cells and their role in the pathogenesis of inflammatory diseases, the crucial role of the IL-17 axis has been shown in the pathogenesis of BD [36–38]. IL-17 is a strong proinflammatory cytokine that mobilized and activate neutrophils and macrophages and may be involved in the pathogenesis of inflammation in acute attacks in BD [39]. Chi *et al.* in a study on 23 patients with BD showed higher expression of IL-17 by peripheral blood mononuclear cells in patients with uveitis compared with patients without uveitis and healthy controls [37]. Another study showed higher production of IL-17 by $CD4^+$ T cells in BD patients with active uveitis compared with BD patients with inactive uveitis and healthy controls [40]. Kim *et al.* found higher Th17/Th1 ratio in BD patients compared with patients with rheumatoid arthritis and healthy controls [41]. They found higher Th17/Th1 ratio in BD patients with uveitis compared with other BD patients.

MiR-146b affects the activity of Treg cells by regulating FoxP3 and RORγt. MiR-146b reduces the level of IL-10 and TGF-β and increases the level of IL-17 [32]. It has been shown that miR-21 and miR-146b are regulated by IL-6 and/or signal transducer and activator of transcription-3 [33,34].

To the best of our knowledge, our study was the first to assess the expression of miR-21, miR-146b and miR-326 in patients with BD. Our results showed high sensitivity and specificity of miR-326 for prediction of uveitis and severe eye involvement in BD. However, our study had some limitations. The number of cases was low. It was a cross-sectional study and could only show the association between clinical manifestations of BD and studied miRNAs. No causality between studied miRNAs and BD can be demonstrated. Differences in miRNA expression levels between BD and healthy control groups could be an indicator for the contribution of other genetic and epigenetic factors involved in the gene expression regulation that was not evaluated in this study.

Conclusion

The present study showed lower expression of miR-21 and higher expression of miR-326 in patients with BD, higher expression of miR-326 in BD patients with uveitis and severe eye involvement and higher expression of miR-21 in BD patients with severe eye involvement. Although the sensitivity and specificity of these miRNAs for diagnosis of BD were low, the sensitivity and specificity of miR-326 for prediction of uveitis and severe eye involvement in patients with BD were high. MiR-326 expression rate measurement can be used as a biomarker for prediction of uveitis and severe eye involvement in patients with BD.

Author contributions

G Jadideslam has performed laboratory analysis, conception and designing of the study, drafting the manuscript. A Khabbazi has participated in the designing of the study, drafting of the manuscript, critical revision of the manuscript for important intellectual content and final approval of the manuscript. M Ghojazadeh and K Ansarin have participated in data analysis and in drafting the

manuscript. E sakhinia, Z Babaloo and A Abhari have participated in supervision of RNA extraction and miRNAs expression rate. S Alipour, J Farhadi and SS Shirvani have provided patients' data and samples and participated in drafting the manuscript. All the authors read and approved the final manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study had been approved by the committee of the Medical Research Ethics of the Tabriz University of Medical Sciences (TBZMED.REC.1395.1357).

Summary points

- Our study included 46 Behcet's disease (BD) and 70 age- and sex-matched healthy controls.
- Underexpression of MiR-21 and overexpression of miR-326 were observed in patients with BD. No significant difference was observed for miR-146b expression.
- MiR-21 and miR-326 expression were high in patients with severe eye involvement and miR-326, overexpressed in eye involvement, severe eye involvement and erythema nodosum. In contrast, the level of expression of miR-146a in patients with erythema nodosum decreased significantly.
- Sensitivity and specificity of miR-21, miR-146b and miR-326 for diagnosis of BD were low.
- The sensitivity and specificity of miR-326 for prediction of severe eye involvement were 92 and 90%.
- MiR-21, miR-146b and miR-326 are not appropriate biomarkers for diagnosis of BD. However, miR-21 and miR-326 can be used as a useful marker for prediction of uveitis and severe eye disease development in patients with BD.

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