

Vitamin D and miRNA-155 in Behçet's Disease: Possible Association with the Disease and Disease Activity

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Abstract

Background: Vitamin D (vit D) controls inflammation and immunity. In Behçet's disease (BD), microRNA-155 is recognized as a significant immune response regulator. We aimed to investigate the role of vit D on immunomodulation and downregulation of inflammatory pathways associated with BD and detect the role of miRNA-155 in BD.

Methods: miRNA-155 expression by Real Time -Polymerase Chain Reaction (RT-PCR), and vit D, nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-κB), and Tumor necrosis fact of TNF-α expression by Enzyme Linked Immunosorbent Assay (ELISA) were assessed.

Results: BD patients had a significantly higher relative expression of microRNA-155 ($P < 0.001$), it was significantly related to vascular manifestations ($P < 0.001$). Vit D relative expression was significantly low in BD ($P < 0.001$). There was a significant rise in miRNA-155 in the active group compared to the inactive group ($P < 0.001$). A significant decrease in vit D levels (IU) was found in inactive and active individuals suffering from BD when compared to controls ($P < 0.001$). A significant rise was found in vit D levels in inactive BD cases ($P < 0.001$). A significant positive correlations were found between miRNA-155, NF-κB, TNF-α, and negative correlations with vit D relative expression in BD patients.

Conclusions: miRNA-155 relative expression is higher in BD is significantly related to vascular manifestations. It may have a relationship to disease activity. Vitamin D relative expression is significantly low in BD patients, which can significantly influence immunomodulatory BD therapy. Vitamin D deficiency linked to active BD.

Keywords: miRNA-155, Reverse Transcriptase Polymerase Chain Reaction Behçet's disease, Vitamin D.

Introduction

Behçet's disease (BD) represents a chronic, autoinflammatory condition affecting multiple organs. Oral, skin, eye, and genital ulcers, as well as vascular and articular abnormalities, are the most prevalent manifestations. While the etiology of BD remains unclear, the function of genetic factors in its progression has been studied (1). Currently, it is understood to be an immunological- related

condition marked by immune system hyperreactivity, which primarily includes the innate immune response. Complex mechanisms are thought to be involved, such as immunologic, environmental, and genetic factors (2). T cells and monocytes are essential because they are the main cell types that enter tissues and cause inflammation in BD patients. Therefore, BD activity is associated with high

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peripheral T cell populations with a proinflammatory shift (3).

Vitamin D's active metabolite is calcitriol (1, 25 (OH)₂ Vitamin D, which affects the regulation of numerous cytokines responsible for immune responses, both directly and indirectly. Patients with autoimmune and inflammatory illnesses frequently have vit D insufficiency (4). Producing TNF- α , interferon (IFN)-interleukin (IL-2), and IL-5 by T helper cells (Th1) cells are suppressed. In contrast, the development of Th2 cells is influenced by vit D. The lack of vit D reduces regulatory T-cells and alters the Th1/Th2 ratio in favor of Th1. In a time- and dose-dependent manner, vit D inhibits the production of mRNA, Toll like receptors (TLR2), and TLR4 proteins in human monocytes (5). Hence, it is suggested that vit D has an immunomodulatory effect on BD.

MicroRNA (miRNA) has been found to have a role in BD through its interaction with other cytokines, such as TNF- α . miRNA-155 and miRNA-146, a and b, are significant immune response regulators among the numerous miRNAs that may control the immune system (6, 7). Behçet's disease (BD) activity is related to a proinflammatory shift in peripheral T cell populations brought on by raised Th17 and Th22 subsets and decreased Tregs. These population shifts could be caused by dysregulation in the miRNAs that mediate the immune response pathways and the formation of effector cells (8). It has been established that miRNA-155 contributes to dendritic cells, B cells, and T cells dysfunction in their development and function. The direct target of miRNA-155 is the transforming growth factor- β -activated kinase 1 binding protein 2 (TAB2), which its expression is elevated in dendritic cells from patients with active BD and may be reduced through miRNA-155 (9).

We intended to test the role of vit D on immunomodulation and downregulation of inflammatory pathways commonly associated with BD and detect the role of miRNA-155 in BD, providing an initial understanding of related molecular mechanisms.

Materials and Methods

Subjects

This work involved 60 cases with BD, at the rheumatology department at Cairo University in Cairo, Egypt between January 2020, and January 2021. All participants were diagnosed using the 1990 International Behçet's Disease Criteria (10), were 30 years old, and were sex-matched with apparently healthy subjects.

They were classified as follows: Group I: 30 healthy individuals; Group II: 30 cases with inactive BD; and Group III: 30 cases with active BD. Activity assessment was performed using the BD current activity form (BDCAF) (11).

Patients over 18 years old and diagnosed with BD met the inclusion criteria. Any individual with overlapping autoimmune disease, cardiovascular disease, nervous system disorders, endocrine, pulmonary, renal, hepatic, or gastrointestinal disorders diagnosed prior to the onset of BD, an established viral, bacterial, mycobacterial, fungal, or other infection at the time of the study, or a history of any cancer in the preceding five years were all excluded.

All processes followed the 1964 Helsinki Declaration and its subsequent revisions. Each subject provided informed consent. The institution's ethical committee approved the project (MS346-2020).

All BD patients had a detailed history taken, a clinical examination, and laboratory testing. BD was classified as mild, moderate, or severe. The severity score was calculated by multiplying each mild symptom by one, each moderate symptom by two, and each severe symptom by three (12).

Blood sampling

Approximately 5 mL of venous blood samples were obtained from all participants using the BD Vacutainer system. The samples were collected in serum separator tubes, allowed to clot for 15 min, and centrifuged at 4,000 \times g for 10 min. Sera were stored at -80 °C until the time of analysis.

RNA extraction

RNA extraction was performed using SinaPure™ RNA (SinaClon BioScience, Tehran, Iran, Cat. No. EX6031), with a protocol for serum total RNA purification, including miRNA, following the manufacturer's instructions. The extracted RNA was subjected to RNA quantitation and purity assessment using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Vilnius County, Lithuania, Cat. No. K0731).

Reverse transcription into complementary DNA

The extracted total RNA in a 20- μ L volume of RT reactions was incubated for 60 min at 37 °C, followed by 5 min at 95 °C using the miScript® II RT Kit (Thermo Fisher Scientific, Vilnius County, Lithuania; Cat. No. 4374966), following the manufacturer's instructions. The complementary DNA (cDNA) of each sample was stored at -80 °C until the next step. miR-155 were quantified by real-time quantitative polymerase chain reaction (RT-QPCR) The thermal cycling profile was as follows: 15 min at 45 °C for cDNA synthesis, followed by 5 min at 95 °C for reverse transcriptase inactivation and polymerase activation. Subsequently, 40 PCR amplification cycles were performed, which involved 15 s of DNA denaturation at 95 °C, 20 s of primer annealing at 55 °C, and 30 s at 72 °C for amplification. Subsequently, the 2- $\Delta\Delta$ Ct method was used to calculate the fold changes of expression or relative quantitation for the target miRNA NF- κ B (Thermo Fisher Scientific cat #85-86083-11), TNF- α (catalog Number:SEA133Ra) levels and vit D (catalog Number:ab213966) expression from blood collected from both healthy controls and BD patients It was assayed by a commercially available Enzyme-linked immunosorbent assay (ELISA), according to manufacturer instructions.

Statistical analysis

SPSS V22 was employed in statistical analysis. The chi-square test was utilized when comparing nonparametric data. $P < 0.05$ was statistically significant. Standard

deviation, mean, and frequency of categorical data were used to summarize the numerical data. ANOVA was utilized to compare more than two groups, while unpaired t-tests were employed to compare two groups. The Pearson correlation coefficient was used to determine correlations between quantitative variables.

Results

This study involved 90 subjects, among which 78 were males (86.6%), and 12 were females (13.3%). They were classified into three groups: 30 healthy control subjects who were gender and age matched (4 females and 26 males); group II: 30 inactive Behçet's patients, 10% of them were females and 90% were males); and group III: 30 active Behçet's patients (83% were males and 17% were females). The age of the control group was 30.86 ± 9.18 years, that of the active Behçet's group was 34.73 ± 11.6 years, and that of the inactive Behçet's group was 35.73 ± 11.6 . The age of onset was 26.6 ± 7.37 years for the inactive group and 25.4 ± 7.48 years for the active group. Disease duration was 9.13 ± 8.3 and 9.33 ± 7.47 for the inactive and active groups, respectively.

Table 1 compares the BD manifestations among active and inactive cases of posterior uveitis, eye manifestations, stroke, and vascular manifestations.

Table 2 compares the relative expression of miRNA-155, vit D levels, C-Reactive Protein (CRP), TNF- α , and NF- κ B levels in the studied groups between active, inactive, and healthy control groups. It shows a statistically significant increase in miRNA-155 relative expression in inactive patients compared to the control group ($P < 0.001$). In addition, a significant increase in miRNA-155 relative expression in active patients compared to the control group ($P < 0.001$), and a significant rise in miRNA-155 in the active group when compared to the inactive group ($P < 0.001$).

Significant decreases in vit D levels (IU) were detected in both inactive and active individuals with BD compared to controls ($P < 0.001$), as well as in patients with active BD compared to inactive BD patients ($P < 0.001$).

Active and inactive BD cases demonstrated a significant increase in CRP levels compared to the control group ($P < 0.001$). Furthermore, there was a significant increase in CRP levels in patients with active BD compared to inactive BD cases ($P < 0.001$). Additionally, a significant increase in the levels of TNF- α was found in inactive and active BD patients

compared to the control group ($P < 0.001$), as well as in patients with active BD compared to inactive BD patients ($P < 0.001$). Moreover, a significant rise in NF- κ B levels was detected in inactive and active BD individuals compared to the control group ($P < 0.001$), and in patients with active BD compared to inactive BD cases ($P < 0.001$).

Table 1. Comparison between the BD manifestations among active and inactive groups.

			Inactive Behçet's	Active Behçet's	P-value
Posterior uveitis	No	Count	11	19	0.039
		%	36.7%	63.3%	
	Yes	Count	19	11	
		%	63.3%	36.7%	
Ocular manifestations	No	Count	30	24	0.01
		%	100%	80%	
	Yes	Count	0	6	
		%	0%	20%	
Stroke	No	Count	30	25	0.02
		%	100%	83.3%	
	Yes	Count	0	5	
		%	0%	16.7%	
Vascular manifestations	No	Count	14	23	0.017
		%	46.7%	76.7%	
	Yes	Count	16	7	
		%	53.3%	23.3%	
Arterial thrombosis	No	Count	25	30	0.02
		%	83.3%	100%	
	Yes	Count	5	0	
		%	16.7%	0%	

$P < 0.05$ is significant.

Table 2. Comparison between levels of miRNA-155, vit D, CRP, TNF- α , and NF- κ B levels in control, active, inactive BD groups.

	Control	Inactive Behçet's	Active Behçet's	P value
miR-155	1.03 \pm 0.05	3.46 \pm 1.03	6.43 \pm 1.76	< 0.001*
VITD (IU)	48.92 \pm 6.8	30.44 \pm 6.26	13.61 \pm 2.45	< 0.001*
CRP (μ g/mL)	1.04 \pm 0.44	4.29 \pm 1.3	21.05 \pm 4.95	< 0.001*
TNF- α (pg/ml)	60.51 \pm 13.88	87.99 \pm 6.03	153.29 \pm 23.34	< 0.001*
NF- κ B (OD450 nm)	141.81 \pm 33.98	214.49 \pm 6.37	356.01 \pm 39.3	< 0.001*

CRP: C-reactive protein; NF- κ B: Nuclear factor- κ B; TNF- α : Tumor necrosis factor α , VITD: vitamin D. * $P < 0.05$ is significant.

miRNA-155, vit D, CRP, TNF- α , and NF- κ B levels in BD cases with vascular affection were compared. CRP was significantly lower in individuals with vascular involvement. BD

cases with arterial thrombosis had significantly lower levels of vit D, CRP, TNF α , and NF- κ B (Table 3).

Table 3. Comparison between levels of miRNA-155, vit D, CRP, TNF- α , and NF- κ B levels concerning vascular affection in BD patients.

Vascular affection			
	No	Yes	P-value
miR-155	4.98 \pm 1.85	4.89 \pm 2.42	0.08
VITD (IU)	20.28 \pm 9.62	24.83 \pm 9.38	0.07
CRP (μ g/ml)	14.52 \pm 9.33	9.69 \pm 8.27	0.046*
TNF- α (pg/ml)	127.2 \pm 37.27	110.09 \pm 34.8	0.08
NF- κ B (OD 450 nm)	299.52 \pm 74.36	262.31 \pm 76.18	0.07
Arterial thrombosis			
	No	Yes	P-value
miR-155	5.09 \pm 2.08	3.42 \pm 1.17	0.08
VITD (IU)	21.28 \pm 9.66	30.26 \pm 6.11	0.047*
CRP(μ g/mL)	13.41 \pm 9.23	4.5 \pm 1.17	0.036*
TNF- α (pg/ml)	123.7 \pm 37.16	86.98 \pm 3.96	0.032*
NF- κ B (OD 450 nm)	291.85 \pm 76.68	212.74 \pm 7.21	0.026*

CRP: C-reactive protein; NF- κ B: Nuclear factor- κ B; TNF- α : Tumor necrosis factor α , VITD: vitamin D. *Statistical significance at P< 0.05.

A significant relationship between the levels of vit D, miRNA-155, CRP, TNF- α , and NF- κ B in the inactive Behçet's group. When vit D levels decrease, the levels of miRNA-

155, CRP, TNF- α , and NF- κ B increase, and vice versa (Table 4) and a significant association between the levels of vit D, miRNA-155, CRP, TNF- α , and NF- κ B in the active Behçet's group.

Table 4. Correlations between the levels of vit D, miRNA-155, CRP, TNF- α , and NF- κ B in inactive BD patients.

		miR-155	VITD	CRP	TNF- α	NF- κ B
miR-155	Pearson Correlation	1	-0.723**	0.752**	0.776**	0.755**
	Sig. (two-tailed)		0	0	0	0
	N	60	60	60	60	60
VITD	Pearson Correlation	-0.723**	1	-0.871**	-0.809**	-0.825**
	Sig. (two-tailed)	0		0	0	0
	N	60	60	60	60	60
CRP	Pearson Correlation	0.752**	-0.871**	1	0.921**	0.893**
	Sig. (two-tailed)	0	0		0	0
	N	60	60	60	60	60
TNF-α	Pearson Correlation	0.776**	-0.809**	0.921**	1	0.945**
	Sig. (two-tailed)	0	0	0		0
	N	60	60	60	60	60
NF-κB	Pearson Correlation	0.755**	-0.825**	0.893**	0.945**	1
	Sig. (two-tailed)	0	0	0	0	
	N	60	60	60	60	60

** Statistical significance at P< 0.01 (two-tailed)

CRP: C-reactive protein; NF- κ B: Nuclear factor- κ B; TNF- α : Tumor necrosis factor α , VITD: vitamin D.

Discussion

The relative expression of miRNA-155 was significantly higher in BD individuals than in controls and was significantly correlated with vascular manifestations. Vit D relative expression was significantly low in BD cases, which could significantly influence immunomodulatory BD therapy. There is a significant positive correlation between miRNA-155, NF- κ B, TNF- α , and vit D relative expression in BD patients.

According to the current findings, individuals with active BD had significantly lower vit D levels than those with inactive stages and healthy controls. This agrees with the results of (13), as they revealed a significant decline in vit D levels in cases with BD. Furthermore, other studies have indicated that vit D is a significant factor in macrophage maturation (14). Additionally, vit D may diminish the Toll like receptor (TLR2), and TLR4 expression on the monocytes surface, impair immunological response, prevent the production of inflammatory cytokines, like TNF- α , and stop TLRs from becoming overactive to suppress inflammation (15). Khabbazi *et al.* discovered that active BD cases had lower vit D levels than the control group. However, these findings did not show a significant relationship between vit D values, disease activity, and significant BD symptoms (16).

Can *et al.* demonstrated that vit D replacement improves endothelial functioning in cases with BD but is not statistically significant (17). Do *et al.* recently observed that vit D substitution significantly reduces the inflammation caused by TLR2 and TLR4 (18).

Intriguingly, we discovered that vit D3 levels in people with inactive BD negatively correlate with the expression of NF- κ B and TNF- α . Using ELISA, this research detected a significant rise in both NF- κ B and TNF- α levels in active and inactive individuals compared to the control group, mainly affecting patients with prominent vascular symptoms and arterial thrombosis. This is consistent with the results of Hayden & Ghosh, where it was found that during inflammatory

responses occurring in BD, nuclear factor kappa B subunits, comprising the NF- κ B complex, are found in an inactive state in the cytoplasm (19). At the same time, NF- κ B activation has been well-described (20).

According to Ji *et al.*, the rs767649 SNP located in the pre-miRNA-155 upstream region disrupts the binding of regulatory factors, such as NF- κ B, to the miRNA-155. This polymorphism alters the miRNA-155 expression (21). Furthermore, Landgraf *et al.* discovered that miRNA-155 expression was elevated aberrantly in a range of activated immune cells, suggesting that miRNA-155 significantly influences the immune response in several autoimmune and autoinflammatory diseases, including BD (22).

Furthermore, it has been demonstrated that miRNA-146a and miRNA-155 expressions are dysregulated in BD (23).

Polymorphisms influence the expression of the miRNA targets in miRNAs, like miRNA-155, and miRNA-146a, and it was found that this contributes to autoimmune disease vulnerability. In BD, the NF- κ B-miRNA-155 axis collaborates with the NF- κ B-miRNA-146a axis to control the duration and severity of inflammation and vascular symptoms, including arterial thrombosis (24). Together with our findings, these results indicated the significance of epigenetic remodeling, like the miRNAs dysregulation, in the pathophysiology and therapeutic response of BD. The present research showed a significant rise in miRNA-155 in the active group compared to the inactive group ($P < 0.001$). This disagrees with an Egyptian study conducted on 84 Egyptian BD patients, which showed no relationship between miRNA-155, and miRNA-146a expression levels and BD activity (25).

The idea that vit D influences immunomodulatory effects in innate immunity-mediated inflammation in BD could be strengthened by the negative association between vit D, miRNA-155, TNF- α , and NF- κ B, as well as the fact that vit D dose-dependently regulates TLRs. Further studies are essential to understand the mechanisms through which vit D

regulates TLR4 and TLR2, the optimal vit D dose required for immune regulation, and whether there are any genetic reasons for why either production of 1, 25(OH)₂D₃ from vit D or signaling via the VDR is altered in some BD patients before vit D can be used clinically as a biomarker of disease activity or as a therapeutic application in BD.

The relative expression of miRNA-155 was significantly higher in BD individuals than in controls and was significantly correlated with vascular manifestations. Vit D relative expression was significantly low in BD cases, which could significantly influence immunomodulatory BD therapy. There is a significant positive correlation between miRNA-155, NF-κB, TNF-α, and negative correlation with vit D relative expression in BD patients. According to the current results, the relative decline in vit D levels is correlated

with an increase in miRNA-155, TNF-α, NF-κB, and CRP levels. This study suggests that miRNA-146a expression and vit D levels in BD patients could play a role in the disease's progression, phenotyping, and development.

Competing Interests

All authors declare no conflict of interests.

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Ethical committee code: MS346-2020. Written consent was taken from all participants.

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