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MicroRNA-199a-5p and Autophagy related gene 5 in Behçet's Disease: Possible Relation and Association with Disease Severity

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Abstract

Behçet's disease (BD) is a chronic inflammatory autoimmune disorder indicated by relapses. MicroRNAs are small RNA fragments that have the potential to regulate gene expression. Autophagy is a defensive and life-sustaining mechanism that involves the targeting of cytoplasmic components to lysosomes for destruction. MicroRNA-199a-5p and autophagy-related gene 5(ATG5) may have a role in BD. Our goal is to assess the miRNA-199a-5p and ATG5 relative expression in BD patients in order to establish if there is any association between their expression levels and the disease severity. Real-time PCR was utilized to evaluate the relative expression of miRNA-199a-5p and ATG5 in blood samples collected from 47 individuals with BD and 50 matched healthy controls. Full history taking and examination of BD patients were performed in addition to the severity index of their disease. There was a highly significant down-regulation of expression of both miRNA-199a-5p and ATG5 in BD individuals than the controls. MiRNA-199a-5p showed a significant decrease in BD patients having thrombophlebitis compared to those who did not have. The relative expression of ATG5 was considerably greater in people with BD who had arterial thrombosis compared to other Behcet sufferers. In BD individuals, there was a strong positive association between miRNA-199a-5p and ATG5 relative expression with r=0.842, and P-value less than 0.001. We concluded that microRNA 199a-5p and ATG5 may have a role in BD; in BD patients, their relative expression was considerably lower than in controls. MiRNA-199a-5p and ATG5 relative expression was significantly associated with thrombophlebitis and arterial thrombosis, respectively.

*Keywords:*MicroRNA (miRNA); Behçet'sdisease; Autophagy

Introduction:

Behçet's disease (BD) is a chronic, recurring inflammatory condition affecting several organ systems. Dermal lesions, ophthalmic, oral ulcers, vaginal ulcers, and articular abnormalities are the most prevalent condition signs. While the exact origin of BD remained unclear, the critical role of genetic factors in its progression has long been recognized **[1].**

MicroRNAs (miRNAs) are non-coding RNA molecules that regulate gene expression by binding to complementary sequences in the coding or the 3' untranslated region (3'UTR) of the target mRNA. miRNAs are capable of regulating cell differentiation, apoptosis, and proliferation **[2].**

MicroRNAs have been embedded in several pathological and physiological processes, and the pathogenesis of many autoimmune disorders as well **[3].** Elmalt et al. revealed that the expression of miRNA-146a was both elevated and related to the activity of the disease in patients with rheumatoid arthritis (RA) **[4].** One of the miRNAs that showed a significant change in its expression in BD is miRNA-199a-5p **[5].** MiRNA-199a-5p and miRNA-199a-3p are derived from two arms of a single miRNA -199 precursor molecules. They were lately suggested as possible cardiac homeostasis regulators **[6]**.

MicroRNA-199a-5p is relevant because it directly focuses on Hypoxia Inducible Factor-1 (HIF-1), a well-characterized transcription factor that regulates

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angiogenesis, primarily by inducing vascular endothelial growth factor (VEGF) transcription **[7].** It affects the pathogenesis of several different types of cancer, including multiple myeloma **[8],** osteosarcoma **[9],** and liver cancer **[10].** By suppressing autophagy and boosting apoptosis, miRNA-199a-5p expression exacerbated vascular endothelial damage **[11].** Reduced miRNA-199a-5p expression resulted in an increase in proinflammatory cytokines [Interleukin-6 (IL-6), IL-15, and leukemia inhibitory factor (LIF)], which may have an essential role in the pathophysiology of chronic inflammatory illnesses like Behçet's disease **[12].**

Autophagy is an intracellular breakdown process in which lysosomes eliminate aggregated proteins, pathogens, and malfunctioning organelles **[13, 14]**. Alterations to the autophagic pathways have been implicated in developing a variety of diseases **[15]**. Autophagy-related gene variants (ATGs) have been implicated in the development of implementing a set of autoimmune disorders, including systemic lupus erythematosus (SLE) **[16]**, psoriasis **[17]**, and Crohn disease **[18]**. Additionally, polymorphism in the ATG5 gene was related to both the gene's expression level and the chance of developing BD **[19].**Sixteen autophagy proteins form the conserved core of the autophagy machinery in all eukaryotes, which catalyzes the production of autophagosomes. The remaining autophagy proteins are organism-specific, and the majority of them are engaged in autophagy regulation or in dictating the specificity of selective autophagy types **[20].**

The goal of this research is to assess the miRNA-199a-5p and ATG5 relative expression in persons with BD in order to discover if there is a correlation between their expression and the BD severity.

Methods:

This study enrolled about 47 individuals with BD who were seen at Cairo University's Rheumatology Department, Cairo, Egypt, and about 50 seemingly healthy people who were age and sex-matched. All individuals involved in this study were diagnosed using the **1990 International Behçet's Disease Criteria. [21].** Adult patients (over 16 years of age and confirmed with BD) met the inclusion criteria. Any overlapping autoimmune disease, proof of cardiovascular disease, nervous system disorders, endocrine, pulmonary, renal, hepatic, or gastrointestinal disorders given a diagnosis prior to the onset of BD, an established viral, bacterial, mycobacterial, fungal, or other infection at the time of the study, and a history of any type of cancer in the preceding five years were all excluded.

All processes followed the 1964 Helsinki Declaration and its subsequent revisions. Each subject provided an informed consent. The project had already been approved by the local department's ethical committee.

All patients with BD underwent a thorough history taking, clinical examination, and laboratory testing. Behçet's disease was classified as mild, moderate, or severe. The severity score was calculated by multiplying each mild symptom by one, moderate symptom by two, and severe symptom by three **[22].**
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Blood sampling

Venous blood (5ml) was taken from patients and control, then collected in EDTA tubes, and stored at -80ºC until RNA extraction.

Detection of miRNA-199a-5p and ATG5 relative expression by QRT-PCR

Total serum RNA's extraction was carried out using the miRNeasy mini kit (Direct-zol™ RNA MiniPrep Catalog No. R2050) following the manufacturer's guidance. RNA quantitation and purity assessment was done using the NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). The COSMO cDNA synthesis Kit (Willowfort-1020500x, UK) was used for reverse transcription (RT) according to the manufacturer's protocol.

The expression of genes was examined by TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7300 Real Time PCR System. The specific sequences of primers are as follows (from 5' to 3'):

MiRNA-199a-5p forward 5'-CCGGGATCCGCAAACTCAGCTTTAC-3' and reverse 5'-CGGAATTCGTGGCGACCGTGATACC-3'

ATG5 forward 5′-GGCCATCAATCGGAAACTCA-3′ and reverse 5′-ACAGGACGAAACAGCTTCTG-3′

GAPDH forward 5'-GAGTCAACGGATTTGGTCGTATTG-3' and reverse 5'-CCTGGAAGATGGTGATGGGATT-3'.

QRT-PCR analysis was conducted according to the manufacturer's credentials (Applied Biosystems, USA). Data were calculated by Sequence Detection Software version 1.7 (PE Biosystems, USA). The relative expression of miRNA-199a-5p and ATG5 were calculated relative to the GAPDH housekeeping gene by the comparative Ct method as stated by the manufacturer recommendations (Applied Biosystems, USA).

Statistical methods

Statistical analysis was performed with IBM® SPSS® Statistics Version 25. Categorical data were presented as frequencies and percentages and were analyzed

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using chi square test. Numerical data were presented as mean and standard deviation (SD). Data were explored for normality by checking the data distribution using Kolmogorov-Smirnov and Shapiro-Wilk tests. Parametric data were analyzed using independent t-test for comparisons between two groups. Correlations between quantitative variables were assessed by Pearson correlation coefficients. Significant P-values are those less than 0.05.

Results

1. Demographic data

The age of BD individuals was 34.36 ± 10.95 years. Their disease duration was 8.89 ± 0.75 years. Around 40 (85.1%) patients with BD were males, and 7 (14.9%) patients were females. The age of control subjects ranged from 20-45 years with a mean of 30.88 ± 6.21 years. While forty-one (82.0%) control subjects were males, and 9 (18.0%) were females. Figure 1 shows the percentage of BD manifestations among cases. Regarding disease severity, the percentages were 12.8% mild cases, 14.9% moderate cases, and 72.3% were severe cases.

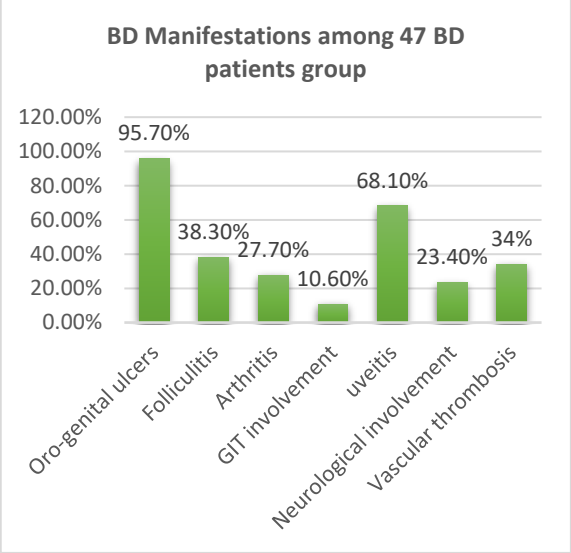

Fig.1 Manifestations of Behçet's disease among the patients.

2. Significant decrease of microRNA-199a-5p and ATG-5 relative expression in BD with no significant difference with disease severity

There was a significant decrease in microRNA-199a-5p and ATG-5 expression in BD cases versus healthy control groups (P<0.001) as shown in (Table 1), however, no significant difference in the expression of these genes was identified among patients with varying degrees of illness severity (p-values were 0.6 and 0.9, respectively) as shown in (Table 2).

Table 1. Relative expression of microRNA-199a-5p and ATG-5 in Behçet's disease versus control

	Control	BD patients	P value
ATG5 Mean± SD	***1.008±0.016***	***0.37±0.22***	***< 0.001****
microRNA 199a-5p	***1.03±0.07***	***0.34±0.12***	***< 0.001****

ATG5, autophagy related gene 5; BD, Behçet's Disease; SD, standard deviation.*the *P*-value ≤ 0.05 is considered significant.

Table 2: Association between microRNA-199a-5p and ATG5 relative expression and BD severity.

		microRNA -199a-5p	P value	ATG5	P value
Severity	**Mild**	***0.39±0.27***	***0.6***	***0.36±0.29***	***0.9***
	moderate	***0.38±0.26***		***0.39±0.25***	
	Severe	***0.32±0.2***		***0.37±0.21***	

ATG5, autophagy related gene 5; BD, Behçet's Disease

3. Relative gene expression of microRNA-199a-5p and ATG-5 in relation to clinical and vascular manifestations

There is no significant difference in microRNA199a-5p relative expression in different clinical data (p-value >0.05) except in thrombophlebitis, where it showed a significant decrease in BD patients having thrombophlebitis compared to those who did not have (p-value <0.001). Also, ATG-5 expression showed no significant difference compared to different clinical data (p-value >0.05) except in arterial thrombosis, where it was significantly higher in patients who had arterial thrombosis than those who did not (p-value <0.001), as shown in (Table 3).

Table 3: Association between gene expression of microRNA-199a-5p & ATG-5 to clinical & vascular manifestations

	microRNA-199a-5p relative expression		ATG5 expression	
Clinical manifestation (%)	Mean± SD	P value	Mean± SD	P value
Oral Ulcer **Yes (95.7%)** **No (4.3%)**	***0.33±0.2*** ***0.53±0.38***	***0.6***	***0.36±0.21*** ***0.62±0.32***	***0.4***
Genital Ulcer **Yes (87.2%)** **No (12.8%)**	***0.35±0.22*** ***0.29±0.17***	***0.5***	***0.36±0.22*** ***0.44±0.19***	***0.3***
Skin affection **Yes (38.3%)** **No (61.7%)**	***0.36±0.26*** ***0.33±0.18***	***0.7***	***0.38±0.22*** ***0.36±0.22***	***0.7***
Arthralgia **Yes (38.3%)** **No (61.7%)**	***0.37±0.26*** ***0.32±0.18***	***0.5***	***0.39±0.24*** ***0.36±0.21***	***0.6***
Arthritis **Yes (27.7%)** **No (72.3%)**	***0.37±0.28*** ***0.33±0.19***	***0.5***	***0.32±0.22*** ***0.39±0.22***	***0.3***
GIT affection **Yes (10.6%)** **No (89.4%)**	***0.47±0.26*** ***0.33±0.21***	***0.2***	***0.25±0.1*** ***0.38±0.23***	***0.07***
Eye affection **Yes (68.1%)** **No (31.9%)**	***0.34±0.2*** ***0.34±0.25***	***0.9***	***0.38±0.22*** ***0.35±0.22***	***0.6***
CNS affection **Yes (23.4%)** **No (76.6%)**	***0.25±0.19*** ***0.37±0.22***	***0.1***	***0.28±0.16*** ***0.4±0.23***	***0.07***
Venous Thrombosis **Yes (34%)** **No (66%)**	***0.28±0.19*** ***0.37±0.22***	***0.19***	***0.45±0.2*** ***0.33±0.22***	***0.07***
Arterial Thrombosis **Yes (8.5%)** **No (91.5%)**	***0.28±0.26*** ***0.34±0.21***	***0.5***	***0.57±0.04*** ***0.35±0.22***	***<0.001******
Thrombophlebitis **Yes (12.7%)** **No (87.3%)**	***0.19±0.04*** ***0.36±0.22***	***<0.001****	***0.48±0.25*** ***0.35±0.21***	***0.19***
Aneurysm **Yes (12.7%)** **No (87.3%)**	***0.36±0.26*** ***0.34±0.21***	***0.8***	***0.53±0.18*** ***0.35±0.22***	***0.06***

ATG5, autophagy related gene 5; BD, Behçet's Disease; GIT, gastrointestinal tract; CNS, central nervous system. **P*-value ≤ 0.05 is considered significant.

4. There was a significant positive correlation between microRNA199a-5p and ATG-5 in BD patients (r=0.842, p-value less than 0.001) as shown in (Fig.2).

Discussion

The purpose of this study was to investigate the relative expression of miRNA-199a-5p and ATG5 in BD people to discover if there is an association between their expression and disease seriousness.

In our study, there was a significant decrease in miRNA 199a-5p gene expression in BD patients compared to the control. This is congruent with the findings of **Antonio et al. [23]**, who examined miRNAs in the blood cells of BD individuals and healthy controls and discovered that 45 miRNAs, including miRNA 199a-5p, were downregulated in BD individuals. **Liang et al. [24],** demonstrated that

when oral lichen planus (OLP) patients' peripheral blood mononuclear cells (PBMCs) were compared to a control group, miR-199 expression was significantly lower in OLP patients. Additionally, **Andrea et al. [25]** found that microRNA-199a-5p expression was dramatically downregulated in psoriatic arthritis which is considered an autoimmune/inflammatory condition.

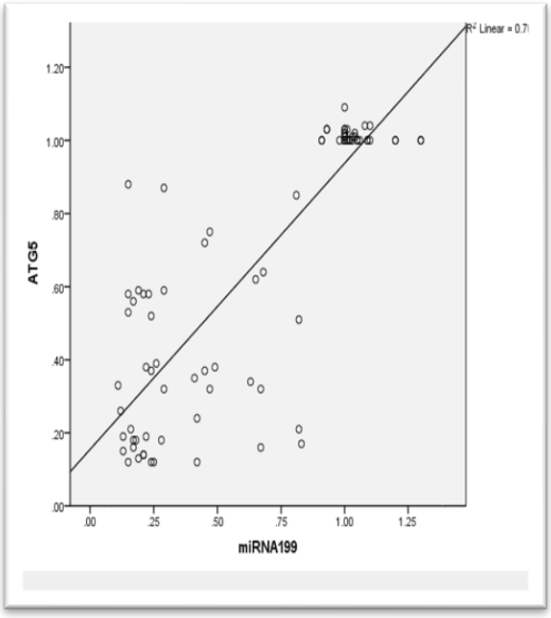

Fig.2 Correlation between microRNA-199a-5p and ATG-5 in BD patients

MiR-199a-5p specifically targets interleukin-6 (IL-6), interleukin-15 (IL-15), and leukemia inhibitory factor (LIF), all of which are major proinflammatory effectors generated by TNF. These compounds were targeted by miRNAs that were down modulated. This was consistent with the miRNAs' traditional role as negative regulators of gene expression (i.e., overexpressed genes are targeted by down-regulated miRNAs) **[12].**In contrast, **Bian et al. [26]**, demonstrated that miRNA-199a-5p expression was increased in patients with active ulcerative colitis compared to controls. Furthermore, miRNA199a-5p expression in the lungs was considerably enhanced in patients with interstitial pulmonary fibrosis (IPF). A high level of miRNA-199a-5p expression was sufficient to augment the pathogenic activation of pulmonary fibroblasts, which included proliferation, invasion, migration, and differentiation into myofibroblasts. Moreover, miRNA-199a-5p regulates caveolin 1 (CAV1), a critical regulator of pulmonary fibrosis in lung fibroblasts **[27].**

In our study, miRNA-199a-5p expression association with disease severity in BD patients revealed no significance. MiRNA199a-5p gene expression was lower in Behcet patients having venous thrombosis and arterial thrombosis with a significant decrease in those with thrombophlebitis compared to those who do not (p-value <0.001). This was consistent with **Lavinia et al. [8]**, who conducted a study using miRNA199a-5p to target multiple myeloma-related angiogenesis and demonstrated that miRNA199a-5p inhibited angiogenesis by down-regulating endothelial adhesion molecules, like intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), as well as the expression of vascular endothelial growth factor A (VEGF-A), IL-8, and basic fibroblast growth factor (bFGF) mRNA. **La Regina et al. [28],** predicted that downregulating miRNA199a-5p would lead to an elevation in ICAM1 and VEGF, which have been seen in the blood of BD patients with a consequent proclivity for thrombosis.

Our investigation discovered that Behçet's patients had significantly lower ATG5 gene expression than controls (P-value less than 0.001). A study conducted by **Mohammad et al. [29]** found no significant downregulation of ATG5, ATG7, mTOR, RAPTOR, or RICTOR in macrophages from BD individuals, but a statistically relevant downregulation of ATG12 and LC3b. On the other hand, **Zheng et al. [19]** found that the level of ATG5 was significantly higher in individuals with active BD than in healthy controls. In a Han Chinese population, this study established a relationship between ATG5 rs573775 and BD.

ATG-5 levels were significantly greater in Behcet individuals with venous thrombosis, thrombophlebitis, and aneurisms than those without (p-value less than 0.001).The vascular effects of ATG5 may be characterized by the decreased ability of mice lacking endothelial ATG5 to produce Von Willibrand Factor (VWF) into the plasma.VWF is required for proper coagulation. Thus, increased ATG5 expression may act as a risk factor for thrombosis **[30].**Thus, autophagy may also play a key role in controlling thrombosis, and autophagy may constitute a novel link between hypercoagulability and endothelial dysfunction **[31].**

In BD patients, our data revealed a substantial positive connection between miRNA199a-5p and ATG-5 gene expression (p-value less than 0.001) which goes with a study done by **Wang et al. [32],**who indicated that miRNA-199a-5p and ATG5 expression was dramatically lowered in the T-cells of individuals with ankylosing spondylitis (AS). TNF-α, IL-17, and IL-23 serum concentrations were increased in AS patients in comparison to healthy controls. Additionally, reduced miRNA-199a-5p phosphorylation of the mechanistic target of rapamycin (mTOR) hindered autophagy.

Limitations of the study

The study has certain limitations: firstly, there is an issue with the tiny sample size. Second, the research objectives were, to some extent, broad in scope due

to a lack of previous research measuring these two parameters in BD. However, in the upcoming research, we need to make the objectives more specific, such as to direct them towards the relationship between the two parameters and the variable vascular manifestations in BD and the correlation of the VEGF as well.

Conclusion

MicroRNA-199a-5p and ATG5 may have a role in BD due to their significantly decreased relative expression levels in BD individuals contrasted to controls.MicroRNA-199a-5p and ATG5 relative expression was significantly associated with thrombophlebitis and arterial thrombosis, respectively. In patients with BD, there was a significant positive relationship between miRNA-199a-5p and ATG5 relative expression.

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Authors' contributions:

Hussein RE*:* participated in supervision of the course of this work and reviewing the article before submission; ***Eissa M****:* participated in writing the manuscript; ***Mohamed DS****:* performed the molecular techniques; ***Gaber RM*** participated in the construction of the hypothesis, contributed to writing the manuscript and corresponding with the journal; All authors have read and approved the final form of the manuscript.

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