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Evaluation the Expression Level of miRNA 155 in Whole Blood as A New Biomarker in the Patients Diagnosed with Alopecia Areata

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ABSTRACT

Background: *Alopecia areata (AA) is a particular type of autoimmune skin illness that causes hair follicles to burst, resulting in abrupt, patchy hair loss that leaves no scars. In rare cases, it may develop to lose hair on the all body (alopecia universalis) or only the hair of the scalp (alopecia totalis). MiRNAs are little non-coding RNAs, approximately less or more than 22 nucleotides in length, and their abnormal function or expression has been involved in many autoimmune diseases. The causes of alopecia areata are not clear yet.* ***Aim:*** *To evaluate the level of miR-155 in whole blood in alopecia areata patients and correlate it with certain clinical variables.* ***Material and methods:*** *miR-155 levels in 40 alopecia areata patients and 40 healthy controls were assessed by using real-time PCR.* ***Results:*** *In comparison to healthy control, the levels of miRNA-155 in the alopecia areata patients differed in a statistically significant way (P < 0.001). There was a negative association between miR-155a and some clinical variables.* ***Conclusion:*** *The pathogenesis of alopecia areata seems to be significantly affected by miR-155.*

Keywords: Alopecia areata, microRNA-155, Real-time PCR.

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1. INTRODUCTION

Alopecia areata (AA) is a specific skin autoimmune illness that damages hair follicles and results in rapid patchy hair loss without scarring and inflammation (1). The clinical manifestation of alopecia areata can differ from localized patches of hair loss to full hair loss on the scalp (Totials) or loss of hair throughout the body (Universalis) (2). Around 2% of people worldwide, regardless of age, gender, or ethnicity, suffer from alopecia areata (3). About 90% of cases with AA affect the scalp, but it can also affect additional parts of the body, including pubic hair, eyelashes, moustache, eyebrows, and beard (4). Although the precise etiology of alopecia areata is still unknown, immunology and genetics are known to be major contributors to the disorder (5).

The endogenous essential transcripts undergo enzymatic cleavage to produce miRNAs, which are small, single-stranded, non-coding RNA molecules of 21–23 nucleotides (6). MiRNAs are circulating extracellular miRNAs found in numerous mammalian cell types (7). Blood, serum, saliva, milk, urine, and cerebrospinal fluid are all places where miRNAs can be found. They may one day serve as biomarkers for various diseases or as treatment targets for AA (7). Around 60% of the target genes in humans and other mammals appear to

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be regulated by miRNAs (8). Emerging indications have shown that miRNAs are important in inhibiting autoimmunity and controlling immunological functions (9).

One of the most common miRNAs that are linked to chronic skin inflammation is miR-155. It is important in controlling innate and adaptive immune responses (10). Some studies demonstrated that miR-155's have a significant role in numerous immune cell types like T cells, B cells, and dendritic cells (11). For instance, miR-155 is crucial for differentiating T-helpers (Th) and maintaining the activity of Th1 and Th17 (12). Furthermore, miR-155 functions as an oncogenic human miRNA and is an essential controller of B-cell development and hematopoiesis (13). Because it can target a wide range of immunological genes, microRNA-155 has been involved in several autoimmune illnesses (10). As a result, any abnormal function of these miRs may contribute to the etiology of autoimmune diseases in humans (14).

The study aims to assess the level of miR-155 in whole blood in alopecia areata patients and correlate it with certain clinical variables.

2. MATERIAL AND METHODS

2.1. Study Design

A case-control study was achieved on the following groups from the first of December 2023 to the first of April 2024. The patients suspected of alopecia areata were diagnosed clinically by physicians in dermatological advisory at Al-Sador Hospital and Al-Najaf Teaching Hospital, Najaf province. Patients with alopecia areata were personally questioned by using an anonymous questionnaire that asked about their gender, age, family history, drug history, severity of diseases, recurrence, and duration of diseases.

2.2. Patients and Controls

The current study included a total of 80 participants, including 40 patients with alopecia areata (24 males, 16 females) and 40 healthy controls (26 males and 14 females). In this study, we included patients with patchy AA of different severity levels in our inclusion criteria, and our exclusion criteria were patients with alopecia areata taking steroid drugs and having systemic diseases.

2.3. Sample Collection

Collect about two milliliters of blood were conveyed to a sterile EDTA tube by using disposable syringes, and then microRNA was taken out from the blood by using the microRNA extraction kit. Then, it can be reserved and extracted of miRNA at -20 °C until analyzed with real-time PCR.

2.4. Measuring the Expression Levels of Mature miRNA-155 by Real-time PCR

Quantitative real-time polymerase chain reaction (Q-PCR), which assesses the relative expression levels of miRNA-155 in the whole blood of patients with AA and controls, was performed by using Applied Biosystems Step One Plus. The housekeeping gene (U6), a reference gene, is likewise measured by real-time PCR by using a one-step approach in both healthy controls and patients.

a. Total RNA Extraction from Whole Blood

By using the kit of Trans Zol Up, mi RNA was extracted from a whole blood sample by the company's instructions,

b. Calculating the Purity and Concentration of Total RNA

A Nanodrop spectrophotometer (THERMO. USA) was used to evaluate the isolated genomic RNA. It measured the absorbance at 260 /280 nm to determine the RNA purity and concentration.

c. cDNA synthesis for miRNA and Housekeep Gene (U6)

The (Qiagen) miScript II RT Kit was used to reversibly transcribe the levels of miR-155 and miR-146a by the manufacturer's instructions.

d. Real-time quantitative PCR

By 1-Step RT-qPCR System permits the finding and relative quantification of RNA expression levels by association’s qPCR Master Mix and miScript Reverse Transcriptase Mix.

• The GoTaq® 1-Step RT-qPCR reaction was made by mixing the RNA template with the qPCR Master Mix, RTMix, MgCl2, PCR primers, (CXR) reference dye, and Nuclease-Free Water, as indicated in Table (1).

• After gently vortexing each solution with the template RNA and all of the reagents thawed on ice, a quick centrifugation was performed to remove any leftover liquid from the tube walls.

Table (1): Reaction mix for RT-qPCR using GoTaq® 1-Step.

Components	Volume	Final Concentration
Mgcl2	1.6 ul	25 mM
GoTaq® qPCR Master Mix	10 ul	1X
miScript™ RT Mix	0.4 ul	1X
Carboxy-X-rhodamine (CXR) reference dye	0.3 ul	1X
Forward Primer (20X)	0.6 µl	300 nM
Reverse Primer (20X)	0.6 µl	300 nM
RNA template	6 µl	0.1 ng-5 µ
RNase-free H2O	up to 20 µl -	

After that, RT-qPCR strip plate tubes were filled with the reaction's ingredients, and an Exispin vortex centrifuge was used to mix the mixture for three minutes and then put into a Miniopticon Real-Time PCR system. Subsequently, RT-qPCR reactions were conducted utilizing the miRNA155 and miRNA146a cycling programmer, as indicated in Table (2).

Table (2): One-step RT-qPCR programs for 155 miRNA and miRNA146a.

Stage	Heat	Period	Rounds
Reverse transcription	37℃	15 min	1
RT inactivation/Hot-start activation	95℃	10 min	1
Denaturation	95℃	10 sec	40
Annealing for miRNA 155	60 C	30 sec	
Annealing for miRNA146a	58℃		
Extension and data collection	72℃	30 sec	

e. Gene Expression Calculation

The Fold changes for miR-155 were calculated by using the comparative Ct method (15), after normalization by using U6 as house gene control.

Statistical analysis

We used means ±S.D. to express the data. SPSS 20 was used for the statistical analysis, followed by the T-test, ROC curve, Chi-square, and correlation. In addition, a P-value below 0.05 was also thought to be statistically significant.

3. RESULTS

This study comprised 40 patients with alopecia areata, and 40 healthy people were the control group. The mean age of the patients with alopecia areata did not differ statistically significantly from that of the healthy control group. The mean age of the AA patients was 27.80 ± 12.64 years, whereas the mean age of the healthy individuals was 25.80 ± 5.69 years (P = 0.364). There were 24 (60.0%) men and 16 (40.0%) women in the patients group and 26 (65.0%) men and 14 (35.0%) women in the control group. Gender did not significantly affect the frequency distribution of participants who were patients or controls (P = 0.644), **Figure 1**. The demographic details of both alopecia areata patients and healthy control subjects are shown in **Table 3**.

Table 3: The demographic features of healthy control participants and alopecia areata patients.

Characteristic	Patients *n* = 40	Healthy control *n* = 40	*P*
Age (years)			
Mean ±SD	27.80 ± 12.64	25.80 ± 5.69	0.364 † NS
Range	5 –65 years	18– 40 years	
< 20, n (%)	10 (25.0%)	7 (17.5%)	0.118 ¥ NS
20-29, *n* (%)	11 (27.5%)	20 (50.0%)	
≥ 30, *n* (%)	19 (47.5%)	13 (32.5%)	
Sex			
Male, *n* (%)	24 (60.0 %)	26 (65.0 %)	0.644 ¥ NS
Female, *n* (%)	16 (40.0%)	14 (35.0%)	

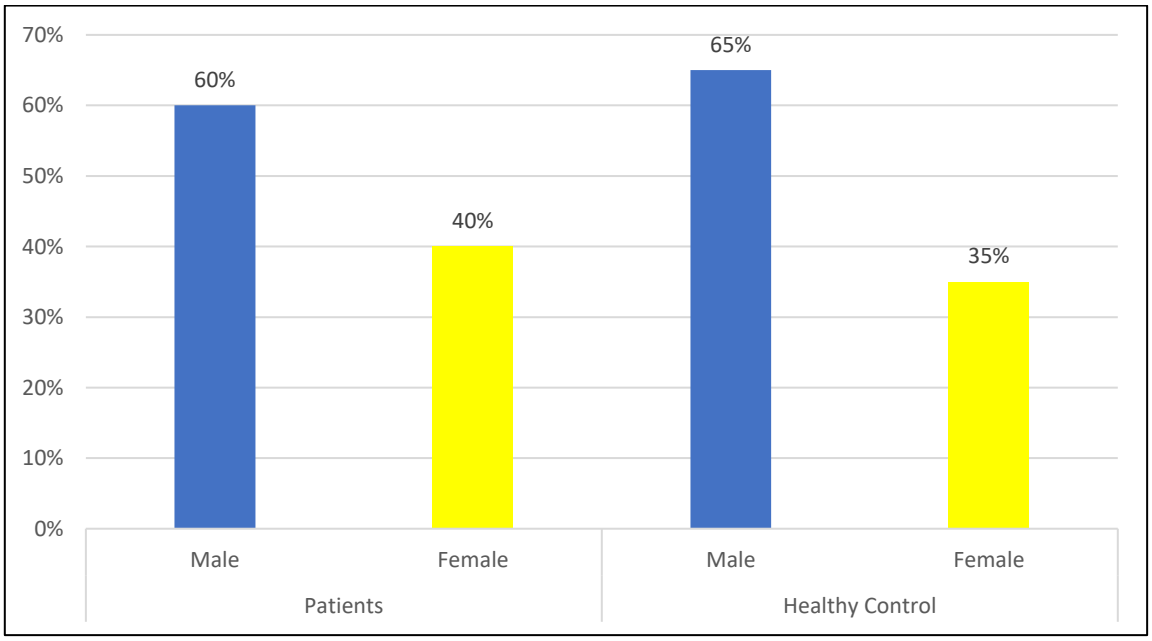

Figure (1): Distribution of patients with AA and control subjects based on sex.

Table (4) and Figure (2) show the outcomes of comparing the levels of miRNA-155 in patients with AA with healthy control subjects. The median levels of miRNA-155 were 4.62 (1.5) in the alopecia areata patients and 0.97 (0.51) in the healthy control group. A statistically significant difference in the levels of miRNA-155 was observed between the two groups (P < 0.001).

The cutoff value of the miRNA-155 and the assessment of alopecia areata disease as a diagnostic was determined using the Receiver Operator Characteristic (ROC) curve analysis. The results are presented in **Table 5**. The cutoff value for miRNA-155 was more significant than 0.995 times, and the sensitivity, specificity, NPV, PPV, and area under the curves were 65.0%, 62.5%, 63.4%, 64.1%, and 0.645 (0.524-0.767), **Figure (3)** . miRNA-155 is thought to be a poor diagnostic marker based on the already available information.

Table (4): miRNA-155 level in healthy controls and alopecia areata patients.

	Comparison of Cases – Control		
	Patients ***n*** **= 40**	**Control** ***n*** **= 40**	***P***
miRNA-155 levels			
Range	0.5-16.10	0.02-6.50	**<0.001** † **HS**
Median (IQR)	4.62 **(1.5)**	0.97 **(0.51)**	

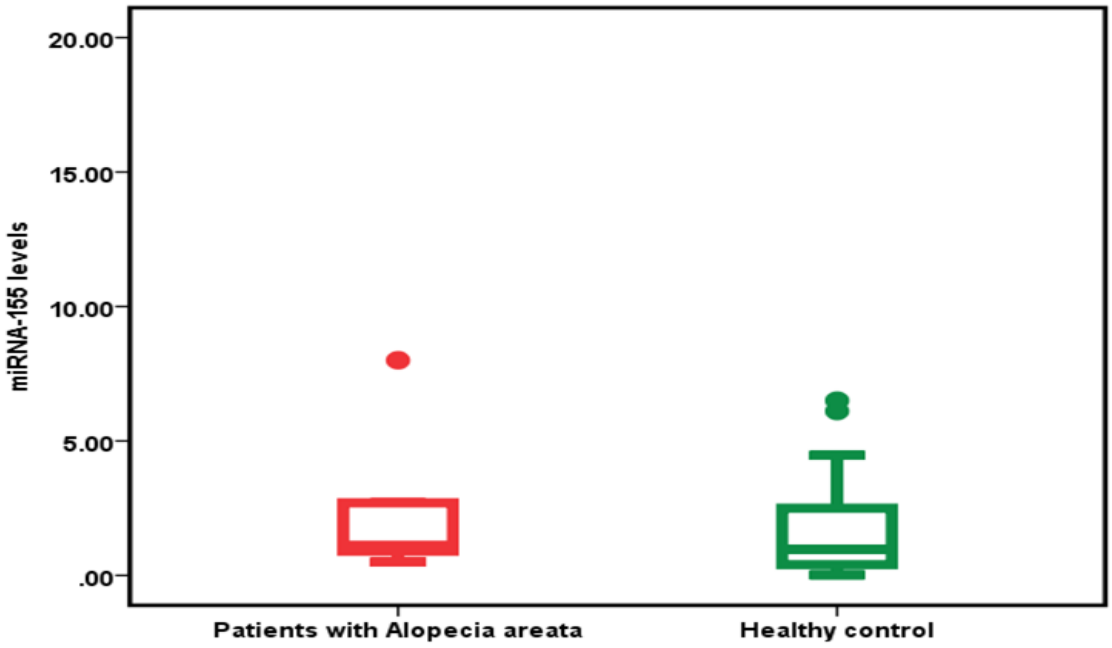

Figure (2): Mean levels of miRNA-155 in both healthy subjects and patients.

Table (5): Alopecia areata disease: Sensitivity and specificity of miRNA-155 level (> 0.995-fold).

miRNA-155 levels	Alopecia areata patients *n* = 40	Healthy control *n* = 40
> 0.995	26 (%)	14 (%)
< 0.995	14 (%)	26 (%)
Sensitivity %	65.0 %	
Specificity %	62.5%	
PPV %	63.4 %	
NPV %	64.1%	
AUC (95% CI)	0.645 (0.524- 0.767)	

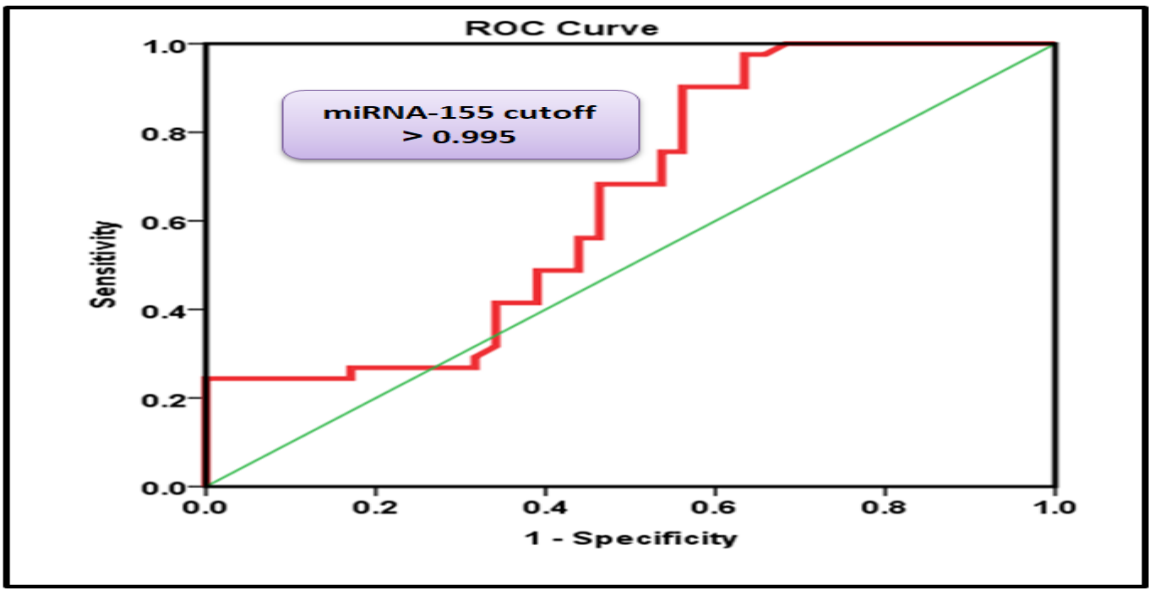

Figure (3): A potential diagnostic cutoff value is found by analyzing the miRNA-155 receiver operator characteristic curve.

MiRNA-155 expression has been compared based on several criteria, and the outcomes are shown in Table (6). The present results show that the miRNA-155 expression was significantly greater in patients with more than four years duration than in other groups (4.46 ± 2.58 vs. 1.74 ± 0.56 and 2.39± 1.43), respectively (P = 0.334). Also, the mean level of miRNA-155 expression was non-significantly greater in patients with a positive family history (3.86 ± 1.81 vs. 1.87 ± 1.46), regardless of the patient's family history (P = 0.130). Additionally, the current findings that there is no significant difference in miRNA-155 expression between patients who have taken drugs in the past and those who have not (2.26 ± 1.15 vs. 2.45 ± 1.99, respectively; P=0.863). Also, in the current results appearance, there was a non-significant difference in miRNA-155 expression according to disease recurrence and severity (P< 0.05).

Table (6): miRNA-155 expression frequency distribution based on certain variables

Characteristics		Mean ± SD	Range	*P*
Duration of disease	< 1 years n=19	1.74 ± 0.56	0.50–11.8	**0.334 A NS**
	1-3 years n=17	2.39± 1.43	0.60 –13.50	
	≥ 4 years n=4	4.46 ± 2.58	0.50-16.10	
Family history	Positive *n* = 8	3.86 ± 1.81	0.50– 16.10	**0.130 † NS**
	Negative *n* = 32	1.87 ± 1.46	0.60- 11.80	
Drug history	Positive *n* = 20	2.26 ± 1.15	0.60 –16.10	**0.863 † NS**
	Negative *n* = 20	2.45 ± 1.99	0.50- 11.80	
Recurrence of disease	Positive *n* =11	2.47 ± 1.33	0.50 – 16.10	**0.898 † NS**
	Negative *n* = 29	2.31 ± 1.31	0.50- 13.50	
Severity of Disease	Mild *n* = 16	2.48 ± 0.98	0.50- 13.50	**0.661 A NS**
	Low *n* = 15	1.88 ± 0.72	0.60- 11.80	
	High *n* = 9	1.41 ± 0.31	0.50- 3.20	

DISCUSSION

There is little research about the function of miRNA in alopecia areata. miRNA can be recognized in numerous sources like whole blood, serum, tissues, and other body fluids, making them a suitable sample for disease biomarkers (16). Alopecia areata (AA) is a condition that causes hair follicles to burst, resulting in abrupt, patchy hair loss that leaves no scars (1). The precise etiopathogenesis of AA has not been known yet, but family history, genetics, organ-specific autoimmune reactions, and emotional stress have all been implicated in the disease (17).

Little non-coding RNA molecules with a length of roughly 20 nucleotides are known as microRNAs or miRNAs. MiRs are significant translational regulators of genes that occur post-transcriptionally because they eliminate or inhibit mRNA translation in a variety of biological processes and tissues (10). They play a critical role in apoptosis, gene regulation, hematopoietic lineage differentiation, and cell proliferation. They also play a role in several human disorders, such as cancer, vascular, immunological, and infectious diseases (18).

One of the most common miRNAs that are associated with chronic skin inflammation is miR-155. It plays a significant part in controlling both adaptive and innate immune responses (10). According to some research, miR-155s play a vital function in many immune cell types, such as B cells, T cells, and dendritic cells (11). Due to its target a wide diversity of immune genes, microRNA-155 has been linked to several autoimmune disorders (10).

Therefore, any deviation in these miRs could have an impact on the genesis of autoimmune disorders in people (14 miR-155 was selected for our investigation to measure blood levels in alopecia areata patients and link them with other clinical parameters. Although Wang *et al*., (2017) discovered an increase of miR-155 in the lesional skin of alopecia areata in mice, their effects on AA patients were not evaluated yet.

In the present study, there was a significant ($P < 0.001$) difference in the amount of miRNA-155 between the alopecia areata patient group and the healthy individual comparison group. According to Sorour *et al*., (2021), patients with AA had significantly higher serum miR-155 levels than controls. These outcomes are agreed with the outcomes of the investigation. The current study verified the ability of miR-155 to predict alopecia areata, which is more significant in predicting AA progression. The present results show that 26 of 40 patients (65%) had AA higher than the cut-off value (> 0.995) in comparison to healthy controls, and 14 (35%) had miR-155 higher than the cut-off value (> 0.995).

According to ROC curve analysis, the miR-155 threshold value was > 0.995, with corresponding levels of 65%, 62.5%, 63.4%, and 64.1% for sensitivity, specificity, PPV, and NPV. The (AUC) was 0.645 (0.524- 0.767). According to the present findings, miRNA-155 is considered a poor diagnostic marker. In a study done by Sorour *et al*., 2021 found the cut-off for miRNA 155 in patients with AA was ≥1.74 and the area under the curve (AUC) was 0.781 and these results are agree with the results of the study.

The current results found there was a non-significant relationship between miR-155 level and duration of disease (p =0.334), family history (p = 0.130), drug history (P= 0.863), recurrence of disease (p = 0.898), and severity of disease (p= 0.661). However, a study by Sorour *et al*., from 2021 discovered that individuals with AA who had recurring disease had much higher serum levels of miR-155 and that there was also a significant positive link between miR-155 levels and the severity of their illnesses.

The benefit of using miRNA as a diagnostic biomarker is its reproducibility and simplicity. A smaller amount of whole blood is required to evaluate the panel of select miRNA. The miRNA has been recognized as a good biomarker for numerous types of diseases because they are constant and may be rising due to the work of a link between tissue and distal cells (19). One of the limits of the study is the same society and small size of the sample, so we need more additional studies and sample sizes to evidence that miRNA is a good biomarker.

CONCLUSION

MiR-155 appears to have a major impact on the pathophysiology of alopecia areata and may serve as a marker for the severity and early recurrence identification of AA. A deeper understanding of the role and control of miR-155 could be highly beneficial in introducing new therapeutic methods for AA.

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