Prognostic markers for severity, activity, and recurrence of alopecia areata
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Background
Alopecia areata (AA) is considered an autoimmune disorder caused by altered T-cell-mediated immunity. MicroRNAs (miR) are important translational regulators of genes in various tissues and biological processes involved in autoimmune responses and have been identified in autoimmune diseases such as rheumatoid arthritis and type I diabetes mellitus. However, little is known about their role in pathogenesis of AA.

Objectives
The aim of this study was to assess serum levels of miR-155 and miR-146a in patients with AA and to correlate them with different clinical variables.

Patients and methods
MiR-155 and miR-146a serum levels were identified by real-time PCR in 50 patients with AA and 50 healthy age-matched and sex-matched patients as controls. Disease severity in the patients was assessed by Severity of Alopecia Tool score.

Results
There was a statistically significant increase in serum miR-155 and miR-146a levels in patients with AA than in controls. A significant increase in serum levels of miR-155 was found in patients with active disease and recurrent lesions of AA, whereas significant increase in serum level of miR-146a was found in patients with recurrent disease only. Significant positive correlations were found between serum miR-155 and miR-146a levels and disease severity.

Conclusion
Serum levels of miR-155 and miR-146a seem to have an essential role in the etiopathogenesis of AA and could be markers for severity and early detection of recurrent AA. In addition, serum miR-155 could be a marker of activity in AA, whereas serum miR-146a could be a marker of multiplicity. Further understanding of the function and regulation of miR-155 and miR-146a could be of great value for the introduction of new therapeutic approaches for AA.

Keywords:
alopecia areata, microRNA-146a, microRNA-155, Severity of Alopecia Tool score

Introduction
Alopecia areata (AA) is an autoimmune disorder characterized by patchy loss of hair from the scalp and other body parts. Alteration in CD4+ T cells, namely, T-helper (Th)1, Th2, and Th17 cells and regulatory T cells, has been implicated in many autoimmune disorders like AA [1].

MicroRNAs (miR) are small noncoding RNA molecules about 20 nucleotides long, and they have emerged as important translational regulators of genes at posttranscriptional level by degrading or blocking translation of mRNA in various tissues and biological processes [2]. They play important roles in cell growth, apoptosis, hematopoietic lineage differentiation, and gene regulation. They are also involved in a wide variety of human diseases such as cancer, vascular diseases, immune diseases, and infections [3].

Previous studies have proved that miR-146a and miR-155 are expressed in many immune cell types such as B and T cells, monocytes, macrophages, and dendritic cells and are two important regulators in both innate and adaptive immunity including the development and differentiation of immune cells, antibody production, and the inflammatory mediator release. Therefore, dysregulation of these miR might participate in the pathogenesis of autoimmune disease in humans [4].

miR-155 has been identified to play a role in the molecular pathogenesis of atopic dermatitis [5] and also has the ability to modulate melanogenesis-associated and interferon-inducible genes in
melanocytes in vitiligo [6]. Both miR-155 and miR-146a have been reported to be significantly elevated in patients with systemic lupus erythematosus [7,8]. Wang et al. [4] found significant upregulation of miR-155 and miR-146a in lesional skin of AA in mice, yet their roles in patients with AA were not assessed.

The aim of this study was to assess serum levels of miR-155 and miR-146a in patients with AA and to correlate them with different clinical variables.

**Patients and methods**

This case–control study was conducted on 50 patients with patchy AA. They were recruited from the Outpatient Clinic of Dermatology and Andrology Department of Benha University Hospitals from January to May 2019. In addition, 50 apparently healthy, age-matched and sex-matched individuals were chosen as a control group. Written informed consent was obtained from each participant. The study was approved by the Local Ethics Committee on Research involving Human Subjects of Benha Faculty of Medicine.

Our inclusion criteria were patients with patchy AA of different degrees of severity according to Severity of Alopecia Tool (SALT) score, age range from 15 to 45 years, and both sexes. All patients enrolled in this study had not received any treatment before or stopped at least 1 month before the beginning of the study.

Our exclusion criteria were patients with metabolic diseases (heart diseases, atherosclerosis, hypertension, and diabetes mellitus), autoimmune diseases (rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus), patients with other causes of hair falling (telogen effluvium and cicatricial alopecia), patients with cancer, and pregnant and lactating women.

**Methods**

All patients were subjected to complete history taking, including personal history (age, sex, occupation, marital status, and residence), present history (onset and duration of AA), past history (of prior episodes of AA and medication history), and family history of AA or other autoimmune diseases. Clinical assessment of the cases including sites, number of lesions was recorded. Severity of AA lesions was assessed using SALT score. SALT score is a mathematical approach to determine percentage of hair loss in the scalp. Scalp is divided into four areas, namely, vertex – 40% (0.4) of scalp surface area (SSA); right profile of scalp – 18% (0.18) of SSA; left profile of scalp – 18% (0.18) of SSA; and posterior aspect of scalp – 24% (0.24) of SSA. Percentage of hair loss in any of these areas is the percentage of hair loss multiplied by percent surface area of the scalp in that area. SALT score is the sum of percentage of hair loss in all the aforementioned areas [9]. Assessment of activity of AA was done by hair pull test [10] and dermoscopic examination (signs of disease activity includes exclamation mark hairs and black dots) [11].

Overall, 3 ml of venous blood was collected from each participant under complete aseptic condition. Blood samples were stored at room temperature for 30 min until clot was formed and then centrifuged for 15 min at 1000g. Serum samples were collected and stored at −20°C until analyzed. Both patients and controls were subjected to determination of serum level of miR-155 and miR-146a with real-time PCR using miScript Primer Assays, containing miR-specific forward primer miR-155 and miR-146a (miRNeasy Mini Kit; Qiagen, Germany).

(1) **Purification of RNA from serum**

Using the miR extraction kits ‘miRNeasy Mini Kit’ (Qiagen), RNAs were isolated from serum according to manufacturer’s instruction.

(2) **Reverse transcription**

Serum miR-155 and miR-146a were reversibly transcribed using miScript II RT Kit (Qiagen) according to manufacturer's protocol. In brief, RNA template was added to the reverse transcription master mix containing five miScript HiSpec Buffer, ×10 miScript Nucleics Mix, miScript Reverse Transcriptase Mix, and nuclease-free water in a total volume of 20 l. The samples were incubated for 60 min at 37°C followed by 5 min at 95°C in conventional PCR thermal cycler (Biometera, Germany) to inactivate miScript Reverse Transcriptase Mix. The undiluted complementary DNA was stored at 20°C till further processing.

(3) **Quantitative real-time PCR**

It was performed following a standard SYBR Green PCR protocol using miScript SYBR Green PCR Kit (Qiagen) with the step one real-time PCR (Applied Biosystems, USA). The real-time cycler was programed, and PCR was done as follows: enzyme activation at 95°C, followed by 40 cycles of denaturation at 94°C for 15 s annealing at 55°C for 30 s, and extension at 70°C for 30 s. The expression of the glyceraldehyde-3-phosphate dehydrogenase
was used as endogenous control for data normalization.

Statistical analysis
The collected data were tabulated and analyzed using SPSS, version 16, software (SPSS Inc., Chicago, Illinois, USA). Categorical data were presented as number and percentages, whereas quantitative data were expressed as mean±SD, median, range, and interquartile range. \( \chi^2 \) test was used to analyze categorical variables. Continuous data were tested for normality using Shapiro–Wilk test assuming normality at \( P \) value more than 0.05, and Student \( t \) test was used to analyze normally distributed variables among two independent groups. Mann–Whitney \( U \) test was used for nonparametric ones. Kruskal–Wallis test was used for three independent groups. Spearman’s correlation coefficient (\( \rho \)) was used for nonparametric variables. \( P \) value was considered significant if less than or equal to 0.05.

Results
The mean age of patients was 32.0±7.6 years, whereas the mean age of controls was 31.0±5.2 years. Patients group included 33 males versus 17 in the control group. There was no statistically significant difference between patients and control groups regarding age and sex (\( P=0.47 \) and 0.11, respectively).

Only four (8%) patients had a positive family history of AA. Clinical assessment of studied patients revealed that five (10%) patients had stable AA and 45 (90%) patients had active AA. A total of 14 (28%) patients presented with single lesions of AA and 36 (72%) patients presented with multiple lesions, and the mean disease duration was 4.6±3.2 months. Results revealed that 31 (62%) patients had AA for the first time, whereas 19 (38%) patients had recurrent AA (all patients gave history of single recurrence).

Regarding the site of AA, three (6%) patients had lesions on the frontal area, three (6%) patients had temporal and/or parietal lesions, three (6%) patients had occipital lesions, two (4%) patients had lesions on vertex area, three (6%) patients had ophiasis, and 36 (72%) patients had multiple areas.

According to results of SALT score, patients were graded into five groups as follows: 56% of patients presented with S1, 12% of patients with S2, 20% of patients represented with S3, whereas S4 was observed only in 12% of patients, and mean SALT score was 33.0±25.9.

The mean serum levels of miR-155 were 3.7±4.5 in patients versus 1.55±1.3 in controls, whereas mean serum levels of miR-146a were 34.2±56.7 in patients versus 1.61±1.36 in controls. There was a significant increase in serum miR-155 and miR-146a levels in patients of AA compared with controls (\( P=0.004 \) and \( P<0.001 \), respectively) (Table 1). A significant increase in serum level of miR-155 was found in patients with active and recurrent AA (\( P=0.027 \) and 0.001, respectively), whereas a significant increase in serum level of miR-146a was found in patients with recurrent AA and multiple lesions (\( P=0.01 \) and 0.001, respectively). There were no statistically significant differences in serum levels of both miR-155 and miR-146a between males and females, between patients with positive family history and those without (Table 2), and between different sites of lesions (Table 3).

Receiver operating characteristic curve was done for the performance of serum miR-146a and miR-155 in prediction of recurrent AA. The AUC were 0.795 and 0.781, respectively, which indicate fair discrimination for both (Table 4).

Serum levels of miR-155 and miR-146a were significantly increased in patients with severe AA (Table 5) and significantly correlated with SALT score (\( \rho=0.558, P<0.001 \) for miR-155 and \( \rho=0.494, P<0.001 \) for miR-146a). There was no statistically significant correlation between serum levels of miR-155 and miR-146a and both age of patients (\( \rho=0.87 \) and \( \rho=0.055, P=0.70 \), respectively) and disease duration (\( \rho=0.137, P=0.22 \) and \( \rho=0.126, P=0.38 \), respectively).

A statistically significant positive correlation was found between serum levels of miR-146a and miR-155 (Fig. 1).

Table 1 Comparison between patients and control groups regarding microRNA-155 and microRNA-146a serum levels

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (N=50)</th>
<th></th>
<th></th>
<th>Controls (N=50)</th>
<th></th>
<th></th>
<th>Z_{MWU} test</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum miR-146a (FC)</td>
<td>34.2±56.7</td>
<td>0.02–200.1</td>
<td>7.5 (0.81–33.5)</td>
<td>1.61±1.36</td>
<td>0.08–4.24</td>
<td>1.52 (0.37–2.62)</td>
<td>4.27</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum miR-155 (FC)</td>
<td>3.7±4.5</td>
<td>0.008–21.23</td>
<td>1.87 (0.86–5.02)</td>
<td>1.55±1.3</td>
<td>0.217–4.35</td>
<td>0.70 (0.43–2.52)</td>
<td>2.89</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

FC, fraction concentration; miR, microRNA; MWU, Mann–Whitney \( U \) test. Significant.
Discussion

Little is known about the functions of miR in AA. They are easily detected in a variety of sources, including tissues, serum, and other body fluids, and this makes them a good biological sample for disease biomarker development [7]. As miR are also stably expressed in body fluid, they have aroused much interest as potential biomarkers to predict disease activity or response to treatment [12].

The results of the present study revealed that serum levels of miR-155 and miR-146a were significantly higher in patients with AA than controls.

As far as we know, this is the first study to assess serum levels of miR-155 and miR-146a in patients with AA. Wang et al. [4] studied the skin expression of miR-155 and miR-146a in mice and found that they were upregulated in the skin samples of five spontaneous...
AA-affected C3H/HeJ mice compared with unaffected C3H/HeJ mice.

Previous studies have proved that miR-146a and miR-155 are expressed in many immune cell types such as B and T cells, monocytes, macrophages, and dendritic cells [4], and their expressions were significantly increased after immunological activation in all four cell types [13].

As AA is one of the autoimmune inflammatory diseases, many inflammatory cells contribute in its pathogenesis as mononuclear cells that accumulate in and around the lesional hair bulb; these cells are CD4+ T cells (60–80%), CD8+ T cells (20–40%), mast cells, dendritic cells, in addition to macrophages, foreign body giant cells, and NK cells [14], which are suggested to be the source of miR.

It is not surprising that this study found increased serum levels of miR-155 in AA, which could be explained by its role on affecting both Th1 and Th2 cells in vitro [15]. The suspected role of miR-155 in pathogenesis of AA could be explained by dysregulation of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is considered as an inhibitor of T-cell activity; the latter has been identified in autoimmune diseases, for example, rheumatoid arthritis in humans [16]. CTLA-4 was found among the predicted and validated targets for miR-155 and is a gene that exhibits a strong genetic association with AA based on previous genome-wide association studies analyses [17]. These data can be in agreement with studies on patients with AA that revealed lower levels of CTLA-4 directly influence the inhibition of Treg and thereby modulate disease susceptibility [18].

miR-155 also might function to block the inhibitory effect of cytokines such as interleukin 4 (IL-4) and interferon gamma (IFN-γ) on the Th17 cell differentiation pathway. miR-155 has been shown to limit production of IL-4 by CD4+ T cells through repression of the transcription factor c-Maf [19]. Previous studies in patients with AA revealed that Th17 cells are infiltrated in dermis and around the hair follicles and can be involved in cell-mediated autoimmunity. In addition, patients with AA have an imbalance between Th17 and Treg cells, with Th17 levels in blood exceeding the Treg levels during active stages of disease; such imbalance can result in inflammation and autoimmunity through similar proinflammatory mechanisms reported in other autoimmune diseases [20].

The suspected role of miR-146a in AA pathogenesis comes from a previous study in AA mice which revealed higher expression of miR-146a in AA skin than controls, resulting in constitutive expression of inducible T-cell costimulator and enhanced T-cell
miR-146a could be a marker of multiplicity. Further understanding of the function and regulation of miR-155 and miR-146a could be of great value for the introduction of new therapeutic approaches for AA.

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Nil.

**Conflicts of interest**
There were no conflicts of interest.

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**References**

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**Results**

of the present study revealed significant associations between miR-155 and miR-146a with patients with recurrent AA and disease severity measured by SALT score, raising the question, could miR be used as prognostic markers for severity of AA and for early detection of recurrent AA? Serum levels of miR-155 are significantly associated with activity of AA, indicating a possible role of miR-155 as an activity marker.

Accumulating evidence from studies points to the contribution of miR-146a in numerous immune processes and in diverse autoimmune diseases, for example, psoriasis [25,26] and rheumatoid arthritis [27,28].

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**Conclusion**

In conclusion, serum levels of miR-155 and miR-146a seem to have an essential role in the etiopathogenesis of AA and could be markers for severity and early detection of recurrent AA. In addition, serum miR-155 could be a marker of activity in AA, whereas serum miR-146a could be a marker of multiplicity. Further understanding of the function and regulation of miR-155 and miR-146a could be of great value for the introduction of new therapeutic approaches for AA.


