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ORIGINAL ARTICLE

Role of Erythroid Differentiation Regulator 1 (Erdr1) in Alopecia Areata

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ABSTRACT

Background: *Several markers have been discovered and validated for alopecia areata (AA). However, their exact roles have not yet been fully studied. Erythroid differentiation regulator 1 (Erdr1), an IL-18-regulated factor, is one of these novel markers.* ***Objectives:*** *This work aimed to study the role of Erdr1 in AA and to correlate its skin mRNA expression level with serum level of IL-18.* ***Methodology:*** *Twenty-five AA patients and 10 healthy control subjects were included in the study. The level of skin expression of Erdr1 mRNA was measured by reverse transcriptase-polymerase chain reaction (RT-PCR). Serum IL-18 level was measured by enzyme-linked immunosorbent assay (ELISA).* ***Results:*** *The level of skin expression of Erdr1 mRNA was 0.214 fold lower in AA patients compared to control group, it was (in log relative unit) 6.61 ± 0.188 Vs 7.17 ± 0.193 for AA patients Vs control group respectively. The level of IL-18 (in pg/ml) in serum was higher in AA patients compared to control group, it was 245.1 ± 51.8 Vs 96.8 ± 38.1 for AA patients Vs control group respectively. In severe type of the disease, there was lower skin level of Erdr1 mRNA and higher serum IL-18 level than in moderate one. There was a significant negative correlation between IL-18 serum level and Erdr1 mRNA skin expression level among patients group (r= -0.786, P <0.001).* ***In conclusion:*** *Erdr1 has a role in the pathogenesis of AA and can be used as a marker for diagnosis.*

INTRODUCTION

Alopecia areata (AA) is non-scarring hair loss resulting from a genetically determined, autoimmune disorder. It is also known as an inflammatory disease of hair follicle cycling[1]. It is characterized by patchy, confluent, or diffuse hair loss which lead to loss of hair on the scalp and other body areas to various extents [2]. The disease affects both sexes and any age. Up to 60 85.5% may experience disease onset before the age of 20 and 40 years, respectively [3].

A wide range of hypotheses exist regarding the etiology and pathogenesis of hair loss disorders. Diverse molecules are involved in the complex pathogenesis of hair loss. For example, autoimmune inflammation, characterized by T-cell predominant infiltrates and associated cytokines such as interferon (IFN)-γ, interleukin (IL)-2, IL-12 and tumor necrosis factor (TNF)-α, were involved in the pathogenesis of AA [4]. In addition, absence of balance between pro-inflammatory and anti-inflammatory cytokines may be the cause of persistent alopecic lesions [5].

IL-18 is an 18-kDa cytokine that belongs to the IL-1 cytokine superfamily. It is known to be an IFN-γ-inducing factor and is a well-known pro-inflammatory cytokine. IL-18 is expressed not only by immune cells but also by non-immune cells like keratinocytes and epithelial cells. Its expression was observed to be increased in different inflammatory cutaneous diseases like psoriasis, rosacea, atopic dermatitis or cutaneous lupus erythematosus[6]. Several studies investigated the pro-inflammatory role of IL-18 in AA [7,8,9,10].

Erythroid differentiation regulator 1 (Erdr1), an IL-18-regulated factor, was first discovered in mouse leukemia cell lines as a hemoglobin synthesis factor. Later on, it was found to modulate cell growth and survival under diverse stressful conditions[11]. Erdr1, a highly conserved autocrine factor, which is distributed widely across different normal human and mouse tissues and is also expressed in normal cells of skin, including keratinocytes and melanocytes[12].

Recently, it has been found that Erdr1 plays an important anti-inflammatory role in different chronic cutaneous inflammatory disorders, like psoriasis and rosacea [13,14]. Also, the pro-apoptotic ability of Erdr1 was confirmed by the recombinant Erdr1 (rErdr1) that found to induce apoptosis of melanoma cells via modulation of apoptosis-regulating factors, like Bcl-2 and Bax, indicating its crucial role as anti-cancer factor in malignant skin cancers like malignant melanoma [15]. However, the exact role of Erdr1 in hair loss disorders is still unclear. This work aimed to study role of Erdr1 in AA and to correlate its skin expression level with serum level of IL-18.

METHODOLOGY

This case–control study was conducted during the period from February 2017 to February2018 in Benha University, Egypt. The work was approved by the Ethics Committee for Human Research of Benha University. Informed consent was taken from all participants.

The study involved 25 Egyptian alopecia areata patients attending the outpatient clinic of Dermatology and Andrology Department, Faculty of Medicine, Benha University, and 10 sex and age matched healthy subjects served as control group.

A standard dermatologic sheet was used to obtain demographic and characteristic information (e.g., age, sex, disease onset, duration and family history of the disease).The severity of the disease was evaluated by dermatologist on the base of the extent of AA: less than 50% involvement of the entire scalp was considered mild-to-moderate AA, and greater than 50% involvement of the entire scalp, alopecia totalis, and alopecia universalis were considered severe AA. Patients with any other auto-immune disease or other active inflammatory skin disease (e.g. vitiligo and psoriasis) were excluded from the study.

Assessment of serum IL-18 level:

Three ml venous blood sample was taken from each patient and control and collected in vacuum tubes. Serum was rapidly separated by centrifugation and then stored at -70°C until processed. Serum IL-18 was quantified by sandwich ELISA technique using a commercial kit developed for the quantitative measurement of human IL-18 in serum [Quantikine® ELISA, Catalog Number DL180]. This assay employs the quantitative sandwich enzyme immunoassay technique

Assessment of skin Erdr1 expression:

Tissue handling:

A 4-mm punch biopsy was taken from scalp skin of each alopecia patient and from equivalent area of each control and stored in RNA later solution (RNA stabilizing reagent) (Qiagen Inc., Valencia, CA) at -80°C for further processing.

Total RNA extraction:

Each stored biopsy was thawed and homogenized using rotor-stator homogenizer (Art-Miccra D-8 Germeny) for 20 sec. Total RNA was extracted using RNeasy mini kit (Qiagen. Inc.,) following the standard protocol. A260 and A280 were taken by UV spectrophotometer (Optima SP-3000+, Japan). Pure RNA has an A260/A280 ratio of 1.9-2.3.

Relative Quantitation (RQ) of mRNA of the respective gene by real time PCR using SYBR green:

A singleplex reaction was used in this work, two-step RT-PCR was done using Maxine RT premix kit (IntRON Bio technology) containing oligo-dT primer and Reverse transcriptase and Real-time-RCR master mix E3 (2X) (Geneon). cDNA synthesis reaction using PCR machine performed at 45°C for 60 min and RTase inactivation step at 95°C for 5 min. In real time PCR instrument ABI7900HT (Applied Biosystems, Foster City, CA, USA), the prepared reaction components were done in 96 well PCR plate using real time cycler conditions of 50°C for 1-2 min, (UNG treatment), 95°C for 1-3 min, (Initial denaturation), followed by 35cycles of 94°C for 30 sec, 62°C for 30 sec and72°C for 1 min for Denaturation, Annealing, Extension steps, respectively. Primer sequence of Erdr1, forward 5′-CAG TGA TGT CAC CCA CGA AA-3′ and reverse, 5′-GGC ATT TCT GTA CGC AGT CA-3′[12]; Primer sequence of Glyceraldehyde Phosphate Dehydrogenase (GAPDH) as internal control (housekeeping gene) was: forward, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′; reverse, 5′-AGC CTT CTC CATGGT GGT GAA GAC-3′. The PCR primers were synthesized by (Operon, inc., Huntsville, Alabama Germany).

Data analysis:

Amplification specificity was checked by melting-curve analysis. The 2 -ΔΔ threshold cycle (Ct) method[16] was used to calculate the relative abundance of target gene expression. The ΔΔ Ct method compares results from Erdr1 gene expression with that of GAPDH gene expression which is housekeeping gene. With this method, Ct is the number of the cycle at which the fluorescent signal of the reaction crosses the threshold. The Ct is used to calculate the initial copy number of the DNA for the studied gene in relation to a normalizer gene. The resulting ΔΔ CT value is incorporated to determine the fold changes in expression.

Statistical analysis:

The collected data were tabulated and analyzed using SPSS version 16 software. Categorical data were summarized in terms of number and percentages, Continuous data were summarized in terms of mean ± standard deviation and range. Data were tested for normality using Shapiro-Wilks test, assuming normality at P>0.05. Differences between groups were tested using Mann Whitney U (ZMWU) test for non-parametric variables. ROC curve was used to detect cutoff values for the studied markers with optimum sensitivity and specificity in prediction of AA. Correlations between variables were assessed using Spearman's correlation coefficient (rho). The accepted level of significance in this work was stated at 0.05 (P <0.05 was considered significant).

RESULTS

The AA patients were 14 males and 11 females with mean age (range) of 21.6±11.5, (11-45). Forty percent of patients have a disease duration > 12 months and 52% of them have disease onset at age ≤13 **y**ears. The disease was mild-to-moderate in 80% of patients while

severe in 20 % of them. Family history was positive in 12 % of patients.

The skin expression level of Erdr1 mRNA was 0.214 fold lower in AA patients compared to control group. The expression of Erdr1 mRNA by log 10 relative units was 6.61 ± 0.188 Vs 7.17 ± 0.193 for AA patients Vs control group respectively. There was a significant decrease in the skin expression level of Erdr1 in AA patients compared with control group *(P <0.001) (Table 1).*

The serum level of IL-18 (in pg/ml) was 245.1 ± 51.8 Vs 96.8 ± 38.1 for AA patients Vs control group respectively. There was a significant increase in the serum level of IL-18 in patients compared with control group *(P <0.001)(Table 1).*

Table (1): Comparison between patient and control groups regarding the mean levels of Erdr1 & IL-18.

Variable	Patient group (n=25)		Control group (n=10)		P value
	Mean ±SD	Range	Mean ±SD	Range	
IL-18 (pg/ml)	245.1±51.8	100-359	96.8±38.1	65-199	<0.001 (HS)
Erdr1 (log10 relative units)	6.61 ± 0.188	6.39-7.22	7.17 ± 0.193	6.64-7.29	<0.001 (HS)

HS=Highly significant. P< 0.05 is significant. n= number.

The skin expression level of Erdr1 mRNA was lower in severe type of the disease than in moderate one. Also, serum level of IL-18 was higher in severe type of the disease than in moderate one. There was a significant relation between both skin expression level of Erdr1 mRNA and serum level of IL-18 and severity of the disease *(P =0.002 and 0.014 respectively*) **(**Table 2).

Table (2): Relation between Erdr1 & IL-18 levels and demographic and clinical variables.

Variable		n.	IL-18 (pg/ml)	P value	Erdr 1 (in relative units)	P value
			Mean ± SD		Mean ±SD	
Sex	**Male**	14	240.5±57.1	0.68 (NS)	679885.3±204853	0.85 (NS)
	Female	11	238± 43.8		597685±215470.5	
Age of onset (In years)	**≤13**	13	238± 58.1	0.93 (NS)	689982±104597.7	0.23 (NS)
	>13	12	251± 37.7		577389.5±119609.5	
Duration (In months)	**≤12**	15	223 ± 33.3	0.3 (NS)	601537.2±128397.4	0.54 (NS)
	>12	10	254 ± 47.5		597881.5±313440.6	
Type	**Mild/ Moderate**	20	236± 37.5	0.014 (S)	798850.2±41807.6	0.002 (S)
	Severe	5	311± 67.1		517037.5±307467.2	
Family history	**Negative**	22	238± 51.8	0.93 (NS)	654375.1±214501.7	0.25 (NS)
	Positive	3	245± 29.7		511346.5±113645.6	

NS=Non significant. S=Significant. P< 0.05 is significant. n= number

No significant relation could be found between both skin expression level of Erdr1 and serum level of IL-18 and each of gender, duration, age of onset or presence of family history of the disease ***(****Table 2).*

There was a significant negative correlation between IL-18 and Erdr1 among patients group *(r= -0.786, P <0.001)* (Figure 1).

The role of Erdr1 as a diagnostic marker of AA was determined and ROC curve was constructed. The ROC analysis showed the best cut off ≤ 6.97 and the corresponding sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and AUC were 96%, 90%, 96%, 90% and 0.950, respectively (Figure 2).

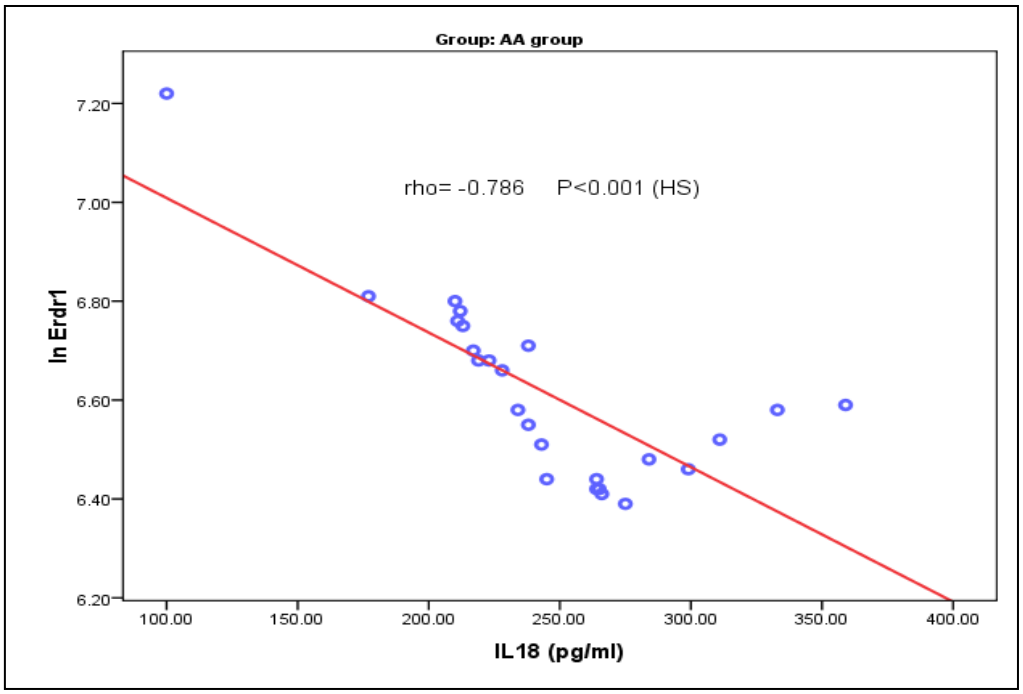

Fig. 1: Scatter plot showing significant negative correlation between IL-18 (pg/ml) and In Erdr1 (In= log transformation of Erdr1) among patient's group.
Obtained using Spearman's correlation coefficient (rho= -0.786, P<0.001 (HS).

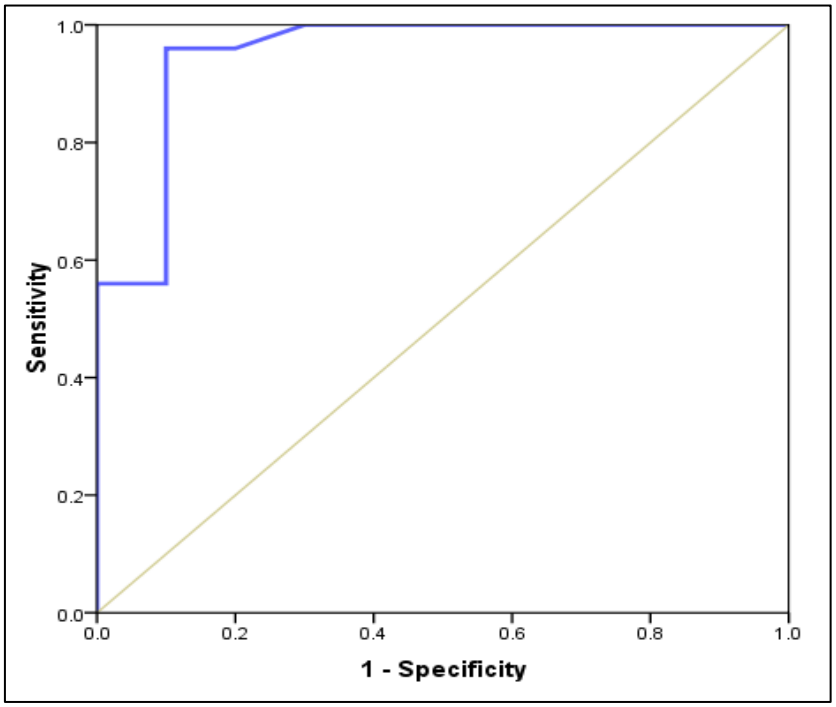

Fig. 2: ROC curve showing excellent ability of Erdr1 as a diagnostic marker of AA.
The best cut off ≤6.97, area under the curve (AUC) = 0.950, 95%CI=0.86-1.0, sensitivity= 96%, specificity= 90%, PPV= 96%, NPV= 90%, Accuracy= 94.3%.

DISCUSSION

Several studies confirmed the anti-inflammatory role of Erdr1 in different chronic inflammatory diseases, like psoriasis and rosacea [13,14], as well as, its anti-cancer effect in malignant skin tumors specially melanoma[15]. In addition, the therapeutic role of Erdr1 have been studied and demonstrated in these diseases [17]. However, the exact role of Erdr1 in hair loss disorders is still unclear. This study was designed to examine the role of Erdr1 in AA. The skin expression level of Erdr1 was measured and the results found that Erdr1 is significantly lower in patients when compared with control group. Indeed, the ROC analysis which represents the ability of Erdr1 as a diagnostic marker of AA was determined and the AUC was 0.950. To the best of our knowledge, there is only one study that analyzes the expression patterns of Erdr1 in different types of alopecias[18], and they also reported Erdr1 downregulation in both scarring alopecia and AA.

In contrast to the Erdr1 downregulation, the results of the current work found a significant increase in the level of IL-18 in the serum of AA patients compared with control group. In agreement with this result, different studies found that the mean level of IL-18 was higher in the patients with AA than in the control group[7,8]. Also, other studies reported that IL-18 single-nucleotide polymorphisms which relate to the increased production of IL-18 have been associated with susceptibility to AA[9,10].

In this work, we studied the relation between Erdr1 and IL-18, and the results determined a significant inverse correlation between them. In accordance, there is a study found that Erdr1 is regulated negatively by IL-18 in mouse melanoma[12]. Also, in another work, it is reported that Erdr1 is negatively expressed relative to IL-18 in psoriatic patients[14].

Woo *et al.* [18] proposed that down regulation of Erdr1 may be involved in the inflammatory cascade in AA and they explained the significant downregulation of Erdr1 expression in AA by these points: First, cell-mediated immunity play the principal role in hair loss disorders[19]. Moreover, T helper 1 cytokines in AA are implicated in the dysregulation of the hair follicle's immune homeostasis [20]. One of these cytokines is IL-18, a pro-inflammatory cytokine, an IFN-γ inducing molecule which is the principle factor that induces AA in a murine model [21].Previous studies reported that Erdr1 is regulated negatively by IL-18 in human and mouse skin tissues suggesting the opposite anti-inflammatory effects of Erdr1 with the pro-inflammatory effect of IL-18[12,14]. Second, Erdr1 downregulation in AA is consistent with results of previous studies, which showed Erdr1 downregulation in chronic inflammatory diseases, like psoriasis and rosacea [13,14], suggesting that inflammation is related to Erdr1 downregulation in hair loss disorders.

In this study, we found a significant relation between the severity of AA and the levels of both Erdr1 and IL-18. The level of Erdr1 was significantly lower in severe type of the disease than in mild/moderate one, however, the level of IL-18 is significantly higher in severe form of AA than in mild/moderate form. In contrast, there is a study reported that the severity of AA was not significantly correlated with the intensity of Erdr1 expression[18]. Also, another study found non-significant differences in IL-18 levels between the patients with extensive and localized AA[8]. So, more studies focused on this association are needed to assess the ability of Erdr1 and IL-18 to be used as markers of severity.

Based on the overall anti-inflammatory effects of Erdr1, several works studied its therapeutic effect on different inflammatory skin diseases, rErdr1 has a therapeutic role on rosacea through inhibition of angiogenesis and infiltration of inflammatory cells [13]. In addition, rErdr1 has a therapeutic role on psoriasis through inhibition of TNF-α secretion, infiltration of inflammatory cells into lesional skin, and chemokine production[14]. Besides these skin diseases, Erdr1 can be a potential part of an effective therapeutic approach for AA, further studies are needed to study this possibility.

In conclusion, Erdr1 has a role in the pathogenesis of AA and can be considered a marker for diagnosis.

Conflict of interest:
The authors have declared no conflicting interest.

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