UL 16 Binding Protein 3 Gene Expression: Does It Have an Association With Alopecia Areata?

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Research note

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Abstract

Objective

Alopecia Areata is one of the most widespread autoimmune diseases affecting both sexes of all ages and across all ethnic individuals. Genetics is considered to be a valuable tool for gaining insight into the disease's pathogenesis. Association with UL 16 Binding Protein (ULBP) genes has been detected with autoimmune disorders.

This study aimed to detect UL-16 Binding Protein -3 (*ULBP3*) gene expression levels in cases with AA and to correlate those levels with the clinical course of the disease**.**

This study included 85 subjects: 55 patients with AA and 30 age- and sex-matched healthy controls. The expression level of ULBP3 mRNA was estimated using Real-Time Polymerase Chain Reaction (RT-PCR).

Results

Levels of ULBP3 mRNA in cases were significantly higher in patients with AA in comparison with controls. Also, there were significant correlations between *ULBP3* mRNA levels and age of patients and disease duration in years.

ULBP3 upregulation in AA enforces the theory that postulates the autoimmune nature of AA and *ULBP3* may be involved in AA pathogenesis and its progression.

Introduction

Alopecia Areata (AA) is an autoimmune skin disorder in which the immune attacks are directed primarily towards the hair follicles [1]. The association between AA and other autoimmune also enforced the assumption that AA itself is an autoimmune disease [2].

The UL16-Binding Proteins (ULBPs) are a unique family of class-1 Major Histocompatibility Complex (MHC)-related molecules that were known by their ability to bind to the Human Cytomegalovirus (HCMV) glycoprotein UL-16. The ULBPs are ligands for the Natural Killer Group 2 D (NKG2D) receptor: an activating receptor expressed by Natural Killer cells (NK) [3].

Engagement of NKG2D/ULBPs evokes the cytotoxic reaction of NK cells through activating the Janus kinase 2, signal transducer and activator of transcription 5, extracellular-signal-regulated kinase mitogen-activated protein kinase and Akt/protein kinase B signal transduction pathways [4].

NKG2D ligands expression is lacking in normal tissues, but their expression is precipitated in response to various stimuli and pathological conditions such as cancers and autoimmune-mediated diseases [5].

In normal hair follicles, *ULBP3* is not active because usually the hair follicle is not visible to the immune system. In genetically predisposed individuals, increased levels of *ULBP3* may have the same influence

on initiating the immune response as in AA and/or becoming induced as part of an inflammatory cascade [6].

The purpose of this study was to assess UL-16 binding protein-3 gene mRNA expression levels in patients with AA and correlate these levels with the clinical course of the disease and disease severity.

Ethics approval and consent to participate. Approval was obtained from the Local Ethical Research Committee, Faculty of Medicine, Menoufia university.

A written informed consent was obtained from every participant before enrollment in the study. For participants below 16 years, a written consent was obtained from one of the parents after detailed description of the study.

Methods

This case-control study was conducted on 85 subjects: 55 patients with AA who were selected during the period from January 2018 to December 2018 in addition to 30 age- and sex-matched healthy controls.

Exclusion Criteria

Any patient with any dermatological disease other than AA, patients with other autoimmune or inflammatory diseases, and patients with recent infectious conditions were excluded.

All cases included were subjected to the following:

A complete history of the enrolled patients was taken and recorded, and they were subjected to general and dermatological examination. Clinical data regarding onset, course, duration of disease, and family of alopecia areata were collected.

Assessment of Disease Severity

a. ***According to Kavak et al.*** [7]., patients were divided into three subgroups:

A1: mild, existence of three or less patches of alopecia with the largest diameter being of 3 cm or less or the disease limited to the eyelashes and eyebrows

A2: moderate, existence of more than three patches of alopecia or a patch larger than 3 cm at the largest diameter but not alopecia totalis or alopecia universalis

A3: severe, alopecia totalis, or alopecia universalis

b. The Severity of Alopecia Tool **(SALT)** score set by the National Alopecia Areata Foundation Working Committee [8] was applied for all cases. Scalps were divided into four areas: the vertex 40%, the right side of scalp 18%, the left side of scalp 18%, and the posterior aspect of scalp 24%.

3 mm scalp punch biopsies, were taken from sites where the disease was active.
Scalp biopsy *specimens* from *healthy* volunteers were included as controls.

All subjects included underwent real-time PCR estimation of ULPB3 mRNA expression levels in scalp tissue.

Sample Preparation

Scalp tissue samples were homogenized in RNeasy Lysis Buffer (RLT lysis buffer) (Qiagen RN ease mini kit, Helden, Germany), RNA extraction was done using Qiagen RNA easy mini kit (Helden, Germany) and stored at – 80°C till analysis time.

Assessing RNA quality and purity was determined by measurement of the absorbance at 260 nm (A260). Absorbance reading should be greater than 0.18 to ensure significance. Ratio greater than 1.6 was accepted (A260/A280). If the purity was lower than 1.5, it re-extraction was done. Total RNA concentration was determined using NanoDrop 2000 UV-Vis spectrophotometer (Thermofisher, Scientific Inc, USA). For cDNA synthesis, the extracted RNA sample was briefly incubated in a genomic DNA wipeout buffer for elimination of genomic DNA then, RNA extract was mixed with QuantiTect Reverse Transcription Kit, including Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and a unique RT Primer, incubated for 1h at 42°C to achieve reverse transcription then 5 min at 95°C to stop reverse transcription then 5 min at 4°C.

The mRNA level of *ULBP3* and Glyceral-3 Phosphate Dehydrogenase (GAPDH) (as an endogenous control), were assessed by cDNA amplification using real-time PCR and Quanti Tect SYBR Green PCR kit with ready-made QuantiTect Primer Assay, Qiagen, Applied Biosystems, USA [9], cycling conditions were as follow: 95°C for 15 min 45 repeated cycles at 94°C for 15s, 55°C for 30s, 72°C for 30s , then 72°C for 10 min for final extension.

For relative quantitation of the results, the Cycle Threshold (the point at which PCR product is first detected above a fixed threshold) was determined for each sample [10]. Each run was completed using a melting curve analysis to confirm the specificity and absence of primer dimers. The analysis was done using applied Biosystem 7500, software version 2.0.1. GAPDH was included to monitor RT-PCR efficiency for all samples.

Statistical Analysis

Statistical analysis was conducted using a personal computer with Statistical Package of Social Science (SPSS) version 20 for windows (SPSS Inc., Chicago, o, Illinois, USA). $P \leq 0.05$ is considered statistically significant. Descriptive statistics were conducted, in which quantitative data were presented in the form of Mean (X), Standard Deviation (SD), and range and were presented in the form of Numbers (N) and Percentages (%); analytical statistics were also conducted, including Mann–Whitney U test (U) and Spearman's correlation coefficient (r).

Results

Patients were 37 (67.3%) males and 18 (32.7%) females with a male: female ratio of 2.1: 1. Their age ranged from 7–60 years with 20.6 ± 12.9 years as a mean ± SD value. Control subjects were 18 (60%) males and 12 (40%) females with a male: female ratio of 1.5:1. Their age ranged from 7–60 years with 23.7 ± 17.8 years as a mean ± SD value. They were comparable regarding sex and age.

Regarding clinical data of studied cases; onset of the disease was acute in 39 (70.9%) cases and was gradual in 16 (29.1%0 cases. Course of the disease was stationary in 30 (54.5%) cases and was progressive in 25 (45.5%) cases. Duration of the disease ranged from 0.5-10 years with 10.4±21.3 years as X±SD value. Family history of AA was positive in 7 (12.7%) cases and was negative in 48 (87.3%) cases. SALT score ranged from 5-90 with 30.8±18.3 as X±SD value. According to Kavak's classification; there were 30 (55%) cases with mild AA and there were 25 (45%) cases with moderate AA.

For mRNA expression levels of *ULBP3* gene, levels were significantly higher in cases compared to controls (U= 7.59, P = 0.001) **(table 1).**

A non-significant relationship between mRNA expression levels of *ULBP3* mRNA and clinical data of studied cases was observed **(table 2).** While a significant positive correlations between mRNA expression levels of ULBP3 gene and age of patients in years (r = 0.28, P =0.038) and disease duration in years (r = 0.373, P = 0.005)**.** Also, there was no significant correlation between mRNA expression levels of the *ULBP3* gene and SALT scores **(Figure 1A&B).**

Discussion

Alopecia areata is an autoimmune disease of non-scarring hair loss of the scalp and/or body with unpredicted progression [11].The pathogenesis of AA is not fully understood. However, genetic and environmental elements are well- known to be major influencers in its development [12]. Many studies have reported an increased frequency of atopic disorders with autoimmune diseases as happens in patients with AA, a fact that added more weight to the autoimmune theory [13]. Approximately 20% of people with AA have a family history of the disease, indicating genetic predisposition. Association analysis has yielded a diversity of involved genes, including MHC, cytokines, and immunoglobulins genes [2].

The aim of this study was to assess the mRNA expression levels of *ULBP3* gene in patients with AA and correlate those levels to the clinical course of the disease and disease severity.

The current work revealed that the mean level of *ULBP3* mRNA expression levels in patient groups was significantly higher than that of the control group; this was in-line with **Jaquelen, Beals.** [14]**,** who found that *ULBP3* was highly expressed in the dermal sheath and dermal papilla of two patients with AA and determined that the upregulation of *ULBP3* may be an essential element in initiating the immune response at the onset of AA and may be a part of the inflammatory cascade.

Petukhova et al. [15], studied *ULBP3* expression within the hair follicle of unaffected scalps and scalps of patients with AA and observed noticeable upregulation of *ULBP3* in AA patients but not in healthy controls nor in those with other inflammatory scalp disorders; it was proposed that the autoimmune destruction in AA may be mediated in part by CD8 + NKG2D + cytotoxic T-cells, whose activation may be induced by the upregulation of *ULBP3* gene in the dermal sheath of the hair follicle.

Moftah et al. [16], also documented higher levels of expression of the *ULBP3* gene in patients with AA compared to healthy control individuals.

In the current study, there was a statistically significant correlation between *ULBP3* gene mRNA expression levels and age of patients and duration of the disease in years while no significant correlation was observed with the SALT score. This was in agreement with **Moftah et al.** [16], who established a significant correlation between *ULBP3* expression levels and age and disease duration in the studied cases.

In an attempt to clarify the relationship between *ULBP3* expression and age, **Christiano et al.** [17], related it to the fact that with age, cellular stress and damage happen more frequently which may induce accelerated expression of such gene.

The statistically significant correlation with the duration of the disease was explained by the implication of ULBP3 in the disease pathogenesis, and chronicity [15].

In the current study, severity of AA was assessed according to Kavak's classification, 30(55%) of cases were having mild alopecia areata and 25(45%) cases were moderate alopecia aerate. **Kavak et al., 2000,** [7] found that in their evaluation of 539 patients with alopecia areata that there were 280 patients (51.9%) were having mild disease, 259 (48.7%) patients were having severe disease. Another study done by **Hoffmann et al. 1996,** [18] on 153 patients with alopecia areata found that 54% had mild disease, 24.9% had moderate disease, and 28.1% had severe disease.

Results of the current study showed that SALT score in studied cases ranged from 5-90 with 30.8±18.3 as a mean ± SD value. This was nearly similar to **Hoffmann et al. 1996,** [18] who conducted his study on 153 patients with alopecia areata and found that average SALT scores were 38.2, ranging from 0%-100% but this is away from results of **Morsy et al. 2018**, [19], who studied 20 patients with AA of and 15 healthy subjects as controls revealed that the mean SALT score for patients was (14.03 ± 13.48). Results of this study showed non- significant relationship between mRNA expression levels and severity of the disease. This was in contrast with results of **Moftah et al., 2016**, [16] who showed a relationship between expression level and severity of the condition.

Conclusion

Upregulation *of ULBP3* gene in AA highlighted the concept of autoimmunity in the explanation of the nature of alopecia areata with suggested involvement of *ULBP3* in its pathophysiology.

Limitations Of The Study

- The main limitation of this study was the small sample.
- Financial burden was one of the reasons of number limitation
- Another limitation was that the patient selection did not reflect the general population, as a single clinic was studied.

List Of Abbreviations

AA: Alopecia Areata

ULBP: UL 16 Binding Protein

ULBP 3: UL-16 Binding Protein -3

RT-PCR: Real-Time Polymerase Chain Reaction

MHC: Major Histocompatibility Complex

HCMV: Human Cytomegalovirus

NKG2D: Natural Killer Group 2 D

NK: Natural Killer cells

SALT: Severity of Alopecia Tool

GAPDH: Glyceral-3 Phosphate Dehydrogenase

mRNA: Messenger RNA

Declarations

Ethics approval and consent to participate:

Approval was obtained from the Local Ethical Research Committee, Faculty of Medicine, Menoufia university.

A written informed consent was obtained from every participant before enrollment in the study. For participants below 16 years, written consent was obtained from one of their parents after detailed description of the study.

Ethical Committee number: 1/2017DERM 546

Consent for publication: No personal information is provided.

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Authors' contributions:

W.S, A.M and E.A: Data collection.

W.S, RA and NT: laboratory investigation.

W.S, RA: Result analysis.

WS, AM, EA, NT and RA: manuscript writing.

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References

1. **Jabbari A, Cerise J, Chen J, Mackay-Wigger J et al.** Molecular signatures define alopecia areata subtypes and transcriptional biomarkers. EBioMedicine, 2016 7:240-247**.**
2. **Barahmani N, de Andrade M, Slusser JP, Wei Q et al.** Human leukocyte antigen class II alleles are associated with risk of alopecia areata. J Invest Dermatol. 2008; 128:240–243.
3. **Sutherland CL, Chalupny NJ, Schooley K, Vanden Bos T et al.** UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J. Immun; 2002. 168: 671-679.
4. **Osterberg A, Nelson R, Yaniv B, Foot R et al.** NK cell activating receptors ligand expression in lymphangioleiomyomatosis is associated with lung function decline. JC/ insight, 2016. 1(16): e87270
5. **Raulet D, Gasser S, Gowen B, Deng W et al.** Regulation of ligands for the NKG2D activating receptor. Annu Rev Immunol, 2013.31:413-441.
6. **Martinez-Mir A1, Zlotogorski A, Gordon D, Petukhova L et al.** Genome wide scan for linkage reveals evidence of several susceptibility loci for alopecia areata. Am J Hum Genet. 2007, 80:316–328
7. **Kavak A, Baykal C, Ozarmagan G, Akar U. HLA in alopecia areata.** Int J Dermatol 2000; 39:589-592.
8. **Olsen EA, Hordinsky MK, Price VH, Roberts JL et al.** Alopecia areata investigational assessment guidelines–Part II. National Alopecia Areata Foundation. J Am Acad Dermatol. 2004, 51: 440-447.
9. **qiagen.com** Sample and Assay Technologies Critical Factors for successful Real- Time PCR brochure 2: 07/2010
10. **Dark H .** Real- time PCR. Clinical chemistry, 2004. SO:1680

11. **Betz RC, Petukhova L, Ripke S, Huang H et al.,** Genome-wide meta-analysis in alopecia areata resolves HLA associations and reveals two new susceptibility loci. Nat Commun; 2015. 6:5966.
12. **Darwin E, Hirt PA, Fertig R, Doliner B et al.** Alopecia areata: Review of epidemiology, clinical features, pathogenesis, and new treatment options. Int J Trichology. 2018. 10:51–60.
13. **Spano F, Donovan JC.** Alopecia areata: Part 1: pathogenesis, diagnosis, and prognosis. Can Fam Physician; 2015. 61(9):751-755.
14. **Jacquelyn K, Beals.** New Study Implicates Autoimmune Mechanisms in Alopecia Areata. Nature Journal; 2010. 466:113-117.
15. **Petukhova L., Duvic, M., Hordinsky, M. David Norris et al.** Genome-wide association implicates T-cell and NK-cell activation pathways in alopecia areata. Nature. 2010. 466: 113–11.
16. **Nayera H Moftah, Rasha AH El-Barbary, Laila Rashed, Marwa Said.** *ULBP3*: a marker for alopecia areata incognita Arch Dermatol. 2016; 308: 415-421.
17. **Christiano, Lynn Petukhova, Madeleine D, Maria H et al.** Genome wide association study in alopecia areata implicates both innate and adaptive immunity. 2010. Nature J; 466:113-117.
18. **Hoffmann R, Wenzel E, Huth A, P van der Steen et al.** Growth factor mRNA levels in alopecia areata before and after treatment with the contact allergen diphenylcyclopropenone. Acta Derm Venereol. 1996;76 (1):17-20. doi:10.2340/00015555761720
19. **Morsy H, Maher R, Negm D.** Correlation between serum IL-17A level and SALT score in patients with alopecia areata before and after NB-UVB therapy. J Cosmet Dermatol. 2018; 00:1–5. https://doi.org/10.1111/jocd.12664

Tables

Table (1): Comparison between cases and controls regarding *ULBP3* mRNA level

	Cases N=55	Controls N=30	Test of significance	P value
***ULBP3* Gene**			U	
Range	1.93–2.98	0.02 – 0.34		
X±SD	2.32±0.18	0.17±0.09	7.59	0.001
	-			HS

U: Mann Whitney U test HS: Highly significant S: Significant N: Number

%: Percentage X: Mean

Table (2): Relationship between *ULBP3* mRNA expression level and clinical data of the studied cases

		ULBP3 mRNA level	Test of significance	P value
		X ±SD		
Gender	Male	2.33±0.20	U	0.871
	Female	2.82±0.12	0.162	
Onset of disease	Acute	2.32±0.19	U	0.709
	Gradual	2.31±0.17	0.373	
Course	Stationary	2.32±0.16	U	0.450
	Progressive	2.31±0.20	0.756	
Family history	Positive	2.27±0.31	U	0.454
	Negative	2.32±0.16	0.749	
Kavak's classification	Mild	2.34±0.17	U	0.118
	Moderate	2.28±0.19	1.56	
	Severe	0		

U: Mann Whitney U test SD: Standard deviation X: Mean

Figures

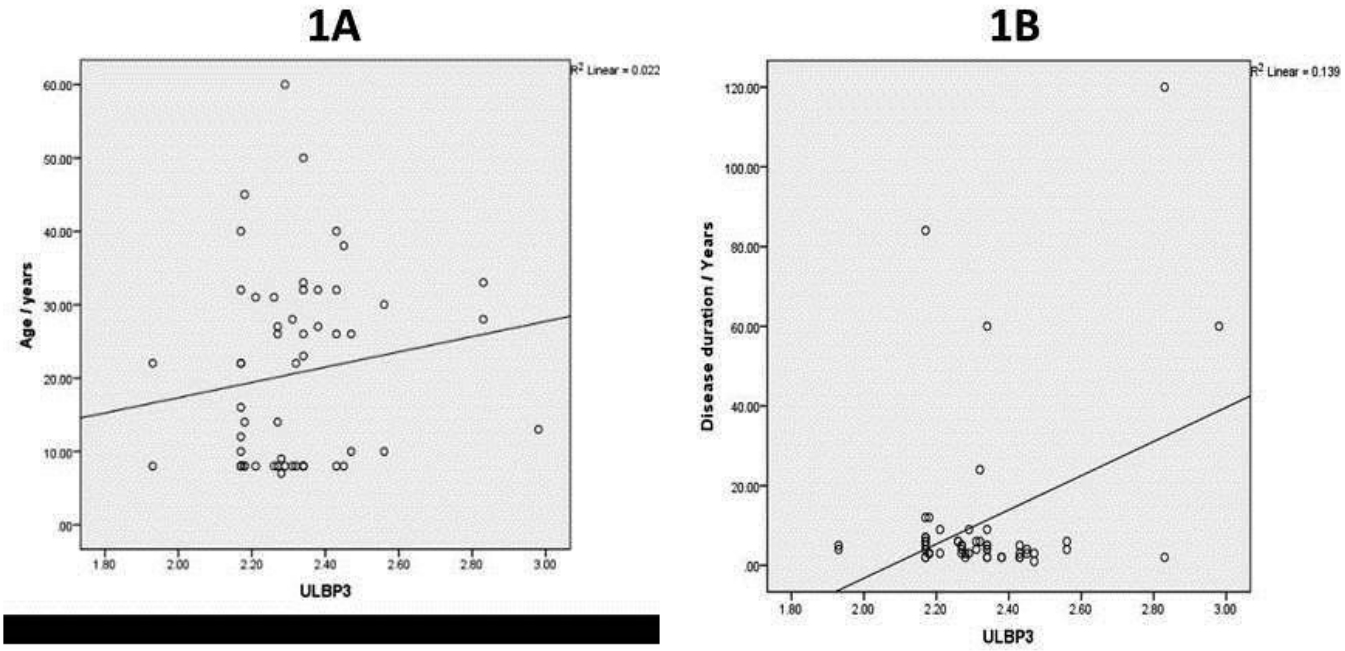

Figure 1

A: Correlation between ULBP3 mRNA expression level and age of patients in years. B: Correlation between ULBP3 mRNA expression level and disease duration in years.