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Interleukin 15 Gene Polymorphism in patients with Alopecia Areata

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ABSTRACT
Background: According to a research done on mice using an IL-15 receptor blocker, there is evidence that IL-15 may be involved in the pathophysiology of Alopecia Areata (AA). It's possible that cytokine gene polymorphisms in the regulatory regions could play a role in immune system illness etiology by altering cytokine concentrations in the body. The aim of the present study was to evaluate the possible association of IL-15 genetic polymorphism (rs17015014) with the risk of development of AA and correlate it with severity of the disease. **Patients and methods:** This case control study was conducted on 50 patients suffering from AA and 50 control subjects. After clinical and dermoscopic examination, severity of AA was measured by SALT score. Detection of interleukin 15 gene polymorphism in DNA extracted from blood sample by polymerase chain reaction and Restriction Fragment Length Polymorphism detection were performed. **Results:** Distribution of IL-15 (rs17015014) gene polymorphism among cases was 22% GG, 60% GC, 18% CC genotypes, while controls had 30% GG, 50% GC and 20% CC genotypes. Cases had 52% G and 48% C alleles, while controls had 55% G and 45% C alleles. No significant association was found regarding genotypes, alleles and AA cases ($p > 0.05$). No significant associations were found regarding IL-15 single nucleotide Polymorphism (SNP) (rs17015014) with SALT scores. **Conclusion**: No associations were found regarding IL-15 genetic polymorphism (rs17015014) with risk of development of AA or disease severity. No associations were found regarding IL-15 genotypes with disease course, recurrence, site of affection or findings of dermoscopy in patients.
Keywords: Alopecia areata, Interleukin-15, Gene Polymorphism, case control study, Mansoura University.

INTRODUCTION
T-cell-mediated autoimmune destruction of hair follicles causes alopecia areata (AA), a non-scarring form of alopecia. AA pathogenesis may be influenced by both hereditary and environmental factors, according to some research [1].

IFN- γ, the primary cause of immune privilege revocation and the emergence of Alzheimer's disease, is produced by NK cells, a major source of interleukin-15 [2]. Patients with AA had significantly elevated levels of serum IL15, which decreased following treatment, supporting the hypothesis that IL15 plays a role in the aetiology of AA [3].

Human chromosome 31 contains the gene for IL-15 (4q31) [4]. Variations in the body's cytokine levels can be caused by genetic polymorphisms in cytokine genes [5]. IL15 gene regulation area polymorphisms may therefore play a role in immune system illness aetiology [6]. AA and IL-15 genetic variation have never been studied together before, to the best of our knowledge.

The aim of the present study was to determine if the IL-15 gene polymorphism is associated with the chance of developing AA, and correlate it with severity of the disease. .

PATIENTS AND METHODS
A case control study involving 50 people with AA and 50 people with no known skin conditions matched for age and gender was carried out. The patients and controls were recruited from Outpatient Clinic of Dermatology at Mansoura University Hospitals. Patients investigated were assessed for illness severity using SALT.

Methods:
The following procedures were performed on all patients:

1. **Taking a complete medical history the history of hair loss in the family,** as well as any other skin conditions or systemic illnesses.
2. **Complete general and dermatological examination.**
3. **Dermoscopic examination:** To confirm the diagnosis.
4. **Grading of AA:**

Severity of AA lesions was assessed using SALT score. Following are the four sections of the scalp: (1) Percentage area: Vertex (0.4) of the scalp. (2) Hairline: 18% (0.18) of the scalp's total surface area is on the right. (3) The left side of the scalp has a surface area of 18 percent. (4) 24 percent (0.24) of the scalp surface area is located on the back of the head. Hair loss percentage in each of these regions is summed together to arrive at a total SALT score [7].

Hair loss was measured as follows: S0, no hair loss. S1, <25% hair loss. S2, 26%–50% hair loss. S3, 51%–75% hair loss. S4, 76%–99% hair loss. S5, total scalp hair loss.

Severity grades were based on the SALT score, and patients were grouped into the following: (1) Mild AA: the S1 group of patients (hair loss less than 25 percent). (2) Moderate AA: Those who were S2 (hair loss from 25 to 49 percent). (3) Severe AA: those patients who were classified as being in the S3 or S4 category, and (hair loss more than 50 percent).

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5. **Laboratory investigations:** Polymerase chain reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) detection of the interleukin 15 gene polymorphism in DNA isolated from blood samples.

Detection of interleukin 15 gene polymorphism:

1. DNA extraction: Blood samples of both patient group and control group were taken and stored at –20ºC into an EDTA containing tube till processed. Genomic DNA was extracted from frozen EDTA-treated blood sample.

Principle: Samples are digested with Proteinase Kinlysis solution. In order to purify the DNA, it is combined with ethanol and placed onto a silica membrane-bound purification column. Impurities are efficiently removed from the column by washing it with the prepared wash buffers. A low-ionic-strength elution solution is then utilized to extract genomic DNA. One hundred microliters of elution buffer were injected into the middle of the column membrane and centrifuged for one minute at 8000xg after two minutes of incubation. Eluted DNA samples were stored at -20°C for future usage when the purification column was removed.

2. Genotyping: IL-15 single nucleotide Polymorphism (rs17015014) for genotyping, a polymerase chain reaction (PCR) was used (PCR). The following reagents were used in the PCR amplification process:

- From the patients and controls, genomic DNA was extracted.
- Primers:
  - Forward: F: 5-CGGACTGCTGGGTCTAAGAAGCTA-3.
  - Reverse R: 5-GTCTGACTCATCAGCCAACACCC-3.
- Taq PCR master Mix:

Protocol: (1) Template and primer solution after being thawed and combined, nucleic acid was ready to be used. (2) It was necessary to defrost and combine the Taq PCRmastermix first. (3) The reaction mixture was gently stirred up and down before being dispensed into a PCR tube for further processing. (4) To begin, the PCR tubes were placed in a thermal cycler, where they were heated to 94C for 30 seconds, annealed at 5- 5C for 30 seconds, and then extended at 72C for 6 minutes.

3. Restriction Fragment length polymorphism (RFLP) for rs17015014:

To separate a 15 microL aliquot of PCR result, we diluted it with 1 microL restriction enzyme and heated it to 37 degrees Celsius for 16 hours.

Ethical consent:

An approval of the study was obtained from Mansoura University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Analysis

In order to analyze the data acquired, Statistical Package for Social Sciences version 20 was used to execute it on a computer (SPSS). In order to convey the findings, tables and graphs were employed. The quantitative data was presented in the form of the mean, median, standard deviation, and confidence intervals. The information was presented using qualitative statistics such as numbers and percentages.

The student's t test (T) is used to assess the data while dealing with quantitative independent variables. Pearson Chi-square (X^2) and Chi-square for Linear Trend were used to assess qualitatively independent data. The significance of a P value of 0.05 or less was determined. Chi-square test was used to identify deviations from expectations based on the Hardy–Weinberg equilibrium (HWE). Gene counts were used to determine the frequency of polymorphisms and genotypes.

RESULTS

The present study was conducted on 50 cases (25 males and 25 females). The average age of the case group was 26 years old (ranged from 3 to 45 years). They were 16 children (32%) and 34 adult (68%). In addition, 50 healthy control subjects, matched for age and gender, were recruited. Among our studied cases, 28% had positive family history and 20% had associated diseases (6% atopic dermatitis, 2% hemolytic anemia, 2% thrombocytopenia, 2% bronchial asthma, 2% hypothyroidism, 2% vitiligo, 2% migraine and 2% had gastric sleeve) **(Table 1)**.

Table (1): Dermoscopic findings among studied cases

Dermoscopic findings	(N=50) n (%)
Yellow dot	27 (54)
Short villous	11 (22)
Broken hair	8 (16)
Tapering hair	3 (6)
Black dot	1 (2)

According to the severity of AA, SALT score of the cases was 1 in 66%, 2 in 18%, 3 in 8% and 4 in 8%. Alopecia severity grades are shown in **Figure I**.

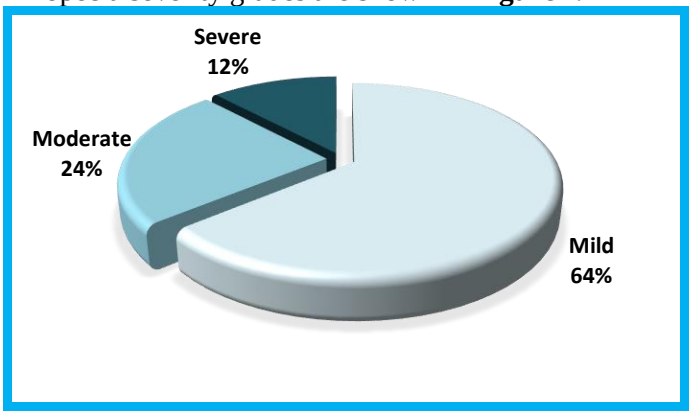

Figure I: Severity grades of alopecia areata patients.

The studied gene (rs17015014) has G and C alleles; G is the reference and C is the alternative. The distribution of different genotypes among cases was 22% GG, 60% GC, and 18% CC genotypes, while controls had 30% GG, 50% GC and 20% CC genotypes. Cases had 52% G and 48% C alleles, while controls had 55% G and 45% C alleles. There was no significant association regarding genotypes, alleles and AA cases (p>0.05 for each) **(Table 2).**

Table (2): Distribution of IL-15 genotypes and alleles among studied groups.

Genotypes	Control N=50		Cases N=50		P value	OR	95% CI
	N	%	N	%			
GG	15	30%	11	22%	-	1	**Reference**
GC	25	50%	30	60%	0.304	1.361	0.756-2.450
CC	10	20%	9	18%	0.736	1.137	0.540-2.391
GC+CC	35	70%	39	78%	0.362	1.299	0.740-2.281
G	55	55%	52	52%	-	1	**Reference**
C	45	45%	48	48%	0.671	1.079	0.761-1.528

OR, odds ratio; CI, confidence interval.

No significant associations were found regarding IL-15 genotypes with family history, disease course, recurrence, body involvement or dermoscopic findings in AA group **(Table 3).** Also, no significant association were found regarding IL-15 genotypes with SALT scores (p > 0.05 for each SALT score or severity grades of AA group (p > 0.05 for each grade). Regarding each genotype, the SALT scores and severity grades did not differ significantly (p >0.05 within each genotype). **(Tables 4 and 5).**

Table (3): Relation of IL-15 genotypes with clinical and dermoscopic findings of alopecia patients.

Genotype	GG (n=11)		GC (n=30)		CC (n=9)		P value
	N	%	N	%	N	%	
Family history							
Negative	6	54.5%	21	70%	9	100%	**0.063**
Positive	5	45.5%	9	30%	0	0%	
Course							
Improving	1	9.1%	4	13.3%	1	11.1%	**0.986**
Stationary	5	45.5%	12	40.0%	3	33.3%	
Progressive	5	45.5%	14	46.7%	5	55.6%	
Recurrence							
First time	7	63.6%	16	53.3%	3	33.3%	**0.451**
Recurrent	4	63.4%	14	46.7%	6	66.7%	
Body involvement							
No body involvement	9	81.8%	26	86.7%	7	77.8%	**0.654**
Body involvement	2	18.2%	4	13.3%	2	22.2%	
Dermoscopy							
Yellow dot	5	45.5%	16	53.3%	6	66.7%	**0.634**
Short villous	3	27.3%	7	23.3%	1	11.1%	**0.659**
Broken hair	3	27.3%	4	13.3%	1	11.1%	**0.507**
Tapering hair	0	0%	2	6.7%	1	11.1%	**0.565**
Black dot	0	0%	1	3.3%	0	0%	**0.712**

Table (4): Relation between IL-15 genotypes and SALT score in alopecia areata patients.

Score		GG (n=11)		GC (n=30)		CC (n=9)		P1
		N	%	N	%	N	%	
SALT score	**1**	9	81.8%	18	60.0%	6	66.7%	0.425
	2	1	9.1%	6	20.0%	2	22.2%	0.672
	3	0	0%	4	13.3%	0	0%	0.235
	4	1	9.1%	2	6.7%	1	11.1%	0.901
	P2	0.511		0.333		0.772		

Chi square was used for comparison. P1, comparison of GG, GC, CC genotypes among SALT grades. P2, comparison of SALT grades among cases carrying specific genotype

Table (5): Association of IL-15 genotypes with severity grades of AA group.

Variable		N	GG n=11		GC n=30		CC n=9		P 1
			N	%	N	%	N	%	
Severity	Mild	32	9	81.8%	17	56.7%	6	66.7%	0.326
	Moderate	12	1	9.1%	9	30.0%	2	22.2%	0.377
	Severe	6	1	9.1%	4	13.3%	1	11.1%	0.929
P 2			0.349		0.393		0.983		

Chi square was used for comparison. P1, comparison of GG, GC, CC genotypes among SALT grades. P2, comparison of SALT grades among cases carrying specific genotype

Alternate models with covariates such as gender and age were used in the ordinal regression analysis in order to predict the severity of AA. None of these factors was considered a predictor of a person's level of AA severity **(Table 6).**

Table (6): Regression analysis for prediction of alopecia areata severity.

	P	OR	95% CI	
Age	0.154	1.031	0.947	1.063
Gender	0.193	1.806	0.906	3.602
Positive family history	0.791	1.107	0.522	2.348
Stress	0.517	0.800	0.407	1.571
Associated diseases	0.672	1.205	0.507	2.864
Progressive course	0.124	0.561	0.269	1.171
Recurrent	0.127	0.455	0.227	1.165
IL15 dominant model	0.219	0.566	0.228	1.402

OR, odds ratio; CI, confidence interval. Ordinal regression analysis was used.

DISCUSSION

Petukhova *et al.* [8] indicated that a person's ancestry plays a role in the development of their disease. Environmental and immunological variables are thought to be as important as a person's genetic background. It's considered a multi-gene autoimmune disorder. **Spano *et al.*** [9] shown an association between AA and some DQB and DR alleles, and the Interleukin (IL)-1 cluster genes.

AA is mainly related to cell mediated immunological factors that results in chronic subclinical inflammation of the hair follicles resulting in hair thinning and falling. These immunological responses are mediated via several cytokines, which are produced from T lymphocytes, they are considered as important players in inducing inflammation [10].

IL-15 is a cytokine that plays a role in both the innate and adaptive immune systems. The anti-apoptotic and pro-apoptotic factors are increased and the pro-apoptotic ones are decreased, respectively, allowing T, B, and NK cells to survive and function. Many different types of cells express it. A number of inflammatory and autoimmune illnesses have been linked to the production of IL-15 in the body. It has been studied for its function in several dermatologic disorders [11] . IFN- γ is produced mostly by natural killer (NK) cells, which are stimulated by the interleukin-15 (IL-15) molecule [12]. That is primarily responsible for the collapse of immunological privilege and the development of AA [2].

There are only a few clinical investigations that have examined the association between IL-15 and illness severity and its involvement in AA, to our knowledge. Therefore, the current study evaluated the association of IL-15 gene polymorphism with the risk of development of AA or disease severity.

In the present study, there were no significant differences between groups regarding age and sex. Median age of the patients was 26 years and ranged from 3 to 45 years with equal sex distribution (25 males and 25 females). Our results agree with **Ghaderi** [13] study that was performed on 50 patients with alopecia, the mean age of them was 29.78 (SD 1.69) however, in their study, 64% were males and 36% were females. While in the study of **Al-Mutairi and Eldin** [14] on AA patients, male to female ratio was 1.86:1 and majority of patients were between 21 and 40 years of age. In a study done by **El-Morsy *et al.*** [15] comprised 39 patients who had scalp AA as their primary complaint, the mean (SD) age of patients was 24.51 (SD 14.26) years. Males comprised 71.8 percent of the 39 patients, while females comprised 28.2 percent.

In our sample of patients, 28% had a history of cancer in their families. This was in agreement with **Esfandiarpour *et al.*** [16] who discovered that 26.5% had a history of AA in their families, and **Wani and Jan** [17] who found positive family history of AA in 20% of the patients. However, in another study, **Manzoor and Masood** [18] found that the family history of AA was low (1.5%). The discrepancy between the results may be attributable to difference in the sample sizes and ethnic differences.

Among our studied cases, 50% had stress. In agreement with our results that stress and phsychological factors have role in developing and aggravating AA, **Alfani *et al.*** [19] reported that patients

with AA appeared to experience more depressive, hysterical and anxiety feelings. Also, **Firooz *et al.*** [20] revealed that 76.9% of people with Alzheimer's disease believed stress was to blame for their illness in research involving 80 participants. Inflammation may be caused by the creation of neuro-mediators capable of interacting with the immune system and causing pathological reactions [21].

SALT scores of 1 in 66%, 2 in 18%, 3 in 8%, and 4 in 8% were found in this study according on the severity grading of AA. This is similar to the work of **Rehman *et al.*** [22], who found a decreasing trend in the severity of AA as measured by the SALT Score: At least 42.62% of the patients had S1 grades (n = 52), followed by 35 in S2 grade, 17 in S3, and 11 in S4 grade, 7 in S5 grade, a total of 35.69%. **Askın *et al.*** [3] discovered a mean SALT score of 71 (SD 30) as a starting point (range, 20-100)

In the current study, 64% of the patients had mild, 24% had moderate and 12% had severe AA. This is in agreement with **Akhyani *et al.*** [23] who studied 239 alopecia individuals in a research. the vast majority of patients (69.9%) suffered from moderate illness, with only 7.5% of patients exhibiting totalis or universalis. Another study done by **Hoffmann *et al.*** [24] with 153 AA patients indicated that 54% had mild disease, 24.9% had intermediate illness, and 28.1% had severe condition.

Dermoscopy, a noninvasive diagnostic technique for AA, has been established. Yellow dots, black dots, short vellus hair, and exclamation mark hair are among the most common dermoscopic findings of AA [25], among our studied cases, 54% had yellow dot. This was in agreement with **Bains and Kaur** [25] who observed yellow dots in 61.5% of cases; Asian individuals may have a yellowish skin tone, which may make the yellow dots more difficult to see. Our percentage of yellow dots was not correlated with severity because most of the present cases were mild. While in another study, **Mahmoudi *et al.*** [26] demonstrated yellow dots in 84.1%.

Non-pigmented vellus hairs may indicate early illness remission. Short vellus hair was discovered in 22% of cases in the current investigation, whereas **Bains and Kaur** [25] found short vellus hair in 57% of the cases studied.

Hairs known as exclamation mark hairs, which are also known as tapering hairs, have a larger distal diameter and a smaller diameter proximal shaft [27]. About 6% of our study had tapering hair, while **Bains and Kaur** [25] found exclamation mark hair in 26.9%.

In the opening of the hair follicle, black spots indicate hair loss and are used to gauge the severity of AA [28]. About 2% of our study had black dot. Dissimilar results were found by **Mane *et al.*** [29] who reported black dots in 67.7% of patients and **Bains and Kaur** [25] who reported black dots in 82.7%.

IL-15 gene polymorphism in AA has never been studied before, to the best of our knowledge. In the present study, cases had 22% GG, 60% GC, 18% CC genotypes, while controls had 30% GG, 50% GC and 20% CC genotypes. Cases had 52% G and 48% C alleles, while controls had 55% G and 45% C alleles. There was no correlation between AA instances and genotypes, alleles, or other genetic factors ($p > 0.05$ for each).

There was no significant associations were found regarding IL-15 genotypes with family history, course, recurrence, site of affection, body involvement and dermoscopic findings in AA group. Likewise, no significant association were found regarding IL-15 genotypes with SALT scores or severity of AA group. Regarding each genotype, no significant difference was found between SALT scores and between severity grades.

Barahmani *et al.* [30] found no significant difference in the IL-15 serum levels of patients compared to those of controls. **Xing *et al.*** [31] JAK inhibitors have been reported to help the disease. A number of mechanisms have been proposed to explain how these medicines work, including inhibiting IL-15-induced pSTAT5 activation and the induction of granzyme B and IFN- γ expression. It appears that IL-15-targeting biologic therapy could be a potential treatment for a wide range of inflammatory disorders in which IL-15 is implicated.

Ebrahim and colleagues [32] found that AA patients had considerably greater levels of blood IL-15 than controls, and there was a strong link between SALT scores and IL-15 levels. This may be an indicator of the severity of the disease. **Askın *et al.*** [3] reported that substantial difference was identified between the baseline IL-15 levels in the patient and control groups. There was no statistically significant association between IL-15 levels at baseline and the severity of the disease (baseline SALT score). Because interleukin levels were elevated in AA patients, the researchers inferred that interleukin plays a role in the disease's development.

In the present study, as covariates in an ordinal regression analysis for the prediction of the severity of AA, IL 15 dominant model and the dominant model of age and gender were used. None was considered as a predictor of AA severity.

CONCLUSIONS

No significant associations were found regarding IL-15 single nucleotide polymorphism (rs17015014) with risk of development of AA or disease severity. No significant associations were found regarding IL-15 genotypes with disease course, recurrence, site of affection or dermoscopic findings in patients. This is the first study to evaluate the role of IL-15 gene polymorphism in AA. Therefore, Interleukin-15 gene polymorphism should be examined in large cohort, which contains different ethnic groups to verify the relationship between IL-15 gene polymorphism and AA and to clarify the clinical significance of IL-15 in AA.

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