





Candidate Gene Analysis Of Alopecia Areata In Jordanian Population Of Arab Descent: A Case–Control Study

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Background: Alopecia areata (AA) is a non-cicatricial patchy hair loss on the scalp, face or other parts of the body. AA was found to be responsive to immunosuppressive therapies, a finding that supports an autoimmune basis for the disease. Several genetic studies have shown the significance of immunological factors as key genetic components in AA.

Objective: In this study, we aimed to investigate the genetic association of 7 single-nucleotide polymorphisms (SNPs) within five candidate genes including *TAP1*, *CXCL1*, *CXCL2*, *HSPA1B*, and *TNF α* with AA susceptibility in the Jordanian Arab population.

Methods: A case–control genetic association study conducted in 152 patients and 150 healthy individuals was performed using the sequenom MassARRAY system (iPLEX GOLD) to genotype the selected SNPs.

Results: rs1800629 SNP of the *TNF α* gene was significantly associated with AA in the heterozygous and rare homozygous genotypes ($P=0.022$ and $P=0.0079$, respectively) with no linkage of the *TAP1*, *CXCL1*, *CXCL2* and *HSPA1B* variants.

Conclusion: This is the first study of its kind among the Jordanian population providing evidence of genetic association of the *TNF α* with AA susceptibility. Further genetic studies on Arab descent including other variants are required to clarify and strengthen the association of these genes with susceptibility to develop AA.

Keywords: alopecia areata, *TAP1*, *CXCL1*, *CXCL2*, genetic association studies, immunological factors, Jordan

Introduction

Alopecia areata (AA) is a disease characterized by areas of non-scarring hair loss that may take the form of a single round, oval patch or even multiple patches that might become confluent. It could affect both men and women equally at any age.¹ Children and young adults are prone more to have the disease, with 30% to 48% of the patients being affected before the age of 20.²

There are several clinical types of alopecia; one favorable division is localized and diffuse types. Localized alopecia can be classified into scarring or non-scarring alopecia.³ Scarring alopecia may be caused by lupus erythematosus, lichen planus, inflammatory tinea capitis, burns or cicatricial basal cell carcinoma. On the other hand, non-scarring alopecia could result from AA, androgenetic alopecia, traction alopecia or non-inflammatory tinea capitis.³

AA is assumed to be a hair-specific autoimmune disease with genetic factors playing a role in disease susceptibility and severity with a concordance rate of 55%

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that is found in identical twin studies implicating both genetic and environmental factors.⁴ The etiopathogenesis of AA is not fully understood yet, due to the multifactorial etiology, whereas several genes and environmental factors play a role in triggering its pathology.⁵

Major histocompatibility complex (MHC) genes have been linked to various autoimmune diseases, including AA.⁶ A transporter 1 ATP-binding cassette subfamily B gene (*TAP1*) is located in the MHC class II region forming a heterodimer that plays a key role in the endogenous antigen presentation pathways. Thus, *TAP* genes represent possible susceptibility for some autoimmune diseases.⁷ Chemokine (C-X-C motif) ligand 1 (*CXCL1*) and chemokine (C-X-C motif) ligand 2 (*CXCL2*) are small cytokines that belong to the CXC chemokine family that plays an important role in modulating T-lymphocyte activities. Therefore, these genes are essential for the pathogenesis of AA as well.⁸ In addition, *HSPA1B* protein is known as heat shock 70-kDa protein 1B. It is involved in modulating antigen processing and presentation and contributes to the immune response against pathogens.^{8,9} In the MHC genes, human leukocyte antigen class III (HLA-III) region contains heat shock proteins such as *HSPA1B*.¹¹ Furthermore, tumor necrosis factor alpha (*TNF α*) is a cell signaling protein (cytokine) that involves in systemic inflammation.¹² *TNF- α* is well known as a major player in the pathogenesis of AA.¹³

This work was focused on non-scarring AA along with alopecia totalis (AT) and alopecia universalis (AU). Our hypothesis pointed to a T-cell-mediated attack on anagen hair follicles after the loss of immune system privilege of the hair follicle which causes the hair to fall in genetically susceptible individuals. Therefore, 7 SNPs within 5 candidate genes were genotyped to reveal the association of AA among the Jordanian population.

Materials And Methods

Participants And Data Source

This study has been established according to the provisions of the Human Ethics Standard and in compliance with the IRB guidelines at Jordan University of Science and Technology (JUST). Approval for patients' recruitment including blood samples and clinical data collection were also obtained from the Human Ethics Committee at The Jordanian Royal Medical Services (RMS) and King Abdullah II University Hospital (KAUH) with a written informed consent obtained from all participants. History of

the disease, clinical diagnosis, medication, treatment follow-up, family history of alopecia and other autoimmune diseases such as thyroid diseases, vitiligo, and psoriasis incidence were obtained.

One hundred and fifty-two patients including 107 males and 45 females were recruited from dermatology clinics of the RMS including Al Hussein Medical City, Prince Rashed bin Al Hasan, Prince Hashem bin Al Hussein, Princes Haya bint Al Hussein and KAUH in addition to 150 unrelated healthy control individuals. The demographic and clinical characteristics of our cohort (302 subjects) are shown in Table 1.

The average age (\pm SD) of the patients' group was 31.144 \pm 12.41 with a median of 31 and a range of 13–67 years. Healthy individuals' age (\pm SD) were 33.9 \pm 9.81 with a median of 32 and the range was 17–64 years. Age groups

Table 1 Demographics And Clinical Data For Alopecia Areata Patients (n=152) And Healthy Individuals (n=150)

Clinical Subtypes Associated With (AA)	Case (n= 152)		Controls (n=150)	
	Number	%	Number	%
Number of Subjects	152		150	
Age (mean \pm SD)	31.144 \pm 12.41		33.9 \pm 9.81	
Gender				
Male	107	70.4%	129	86%
Female	45	29.6%	21	14%
Age of Onset (mean \pm SD)	27.328 \pm 12.57			
< 30 Years	87	57.3%		
\geq 30 Years	65	42.7%		
Type of AA				
Patchy alopecia (PA)	137	90.13%		
Alopecia totalis (AT)	5	3.28%		
Alopecia universalis (AU)	10	6.57%		
Location of AA				
Scalp	92	60.5%		
Face	35	23.02%		
Scalp + Face	8	5.3%		
Other body parts	4	2.63%		
Nail Changes Associated with AA				
(-)	141	92.7%		
(+)	11	7.3%		
Symptoms Associated with AA				
A Symptomatic	104	68.4%		
Symptomatic	48	31.6%		

for patients and control were divided into 3 categories, and each category had a range of 18 years. The first category was (13–31), the second category was (32–50), and the third one was (51–69).

DNA Extraction

Genomic DNA was extracted from EDTA blood samples using commercially available Wizard[®] Genomic DNA Purification Kit (Qiagen, Germany) according to the manufacturer's protocol.

Candidate Genes And SNPs Selection

In order to detect SNPs that could be associated with AA among the Jordanian population, 5 candidate genes (*TAP1*, *CXCL1*, *CXCL2*, *HSPA1B*, and *TNF α*) were selected based on previous association studies or for their position to guarantee the coverage of the entire gene. *TAP1* selected SNPs were rs2071480, rs1135216 and rs1057141. For the *CXCL1* and *CXCL2* chemokines genes, rs3117604 and rs3806792, respectively, rs2763979 for the *HSPA1B* and rs1800629 for the *TNF α* . SNPs were selected using available genetic databases such as the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/SNP/>), the US National Library of Medicine (<https://ghr.nlm.nih.gov/>), and The University of California Santa Cruz Genome Browser Database (UCSC) (<http://genome.ucsc.edu/>).

Genotyping

Extracted DNA samples were sent to be genotyped in collaboration with the Australian Genome Research Facility (AGRF). The Sequenom MassARRAY[®] system (iPLEX GOLD) (Sequenom, San Diego, CA, USA) was used for genotyping at the AGRF based on the manufacturer's procedure. Samples were genotyped in duplicate

and results were accepted unless the success rate was at least 95%.

Statistical Analysis

Hardy–Weinberg equilibrium (HWE) was calculated to estimate genotyping frequencies including examination for ascertainment bias. The probability of evolutionary event existence that may affect the allele frequency was examined, where *p* represents the frequency of one allele, while *q* represents the frequency of the other allele.¹⁴ Any significant deviations from HWE may point to a genotyping error. To define the *P*-values for the allele and genotype association, chi-square test (Pearson χ^2 test) was used to test whether there are any significant differences between the expected, seen and calculated genotype frequencies. Genotypic and allelic association was performed to investigate SNP association with AA. Furthermore, haplotype was performed for linkage disequilibrium (LD) analysis. *P*-value<0.05 was considered statistically significant. The Statistical Package for the Social Sciences (SPSS) version 21.0 and the SNPStat web tool (<https://www.snpstats.net/start.htm>) were used.

Results

In this study, the age of onset (\pm SD) was found to be 27.328 \pm 12.57 with no significant difference in age and gender between patients and controls (data not shown). Our data showed that about 90.13% of the patients had patchy alopecia areata (PA), 6.57% had AU, and only 3.28% of them had AT. Symptoms associated with AA were revealed in 31.6% of the patients, while 68.4% of them had no symptoms at all. Furthermore, only 7.3% of the patients had nail changes associated with the disease. All polymorphisms met the HWE standards in both case and control groups with minor allele frequencies shown in Table 2. Genotyped SNPs were evaluated (presented in

Table 2 Genes, SNPs, Their Minor Allele Frequencies And HWE *P*-Values For Cases And Controls

Gene	SNP	Control (n= 150)			Case (n= 152)		
		MA	MAF	HWE P-value	MA	MAF	HWE P-value
<i>TAP1</i>	rs2071480	A	0.49	0.1	C	0.47	0.87
	rs1135216	C	0.14	0.31	C	0.11	0.4
	rs1057141	C	0.14	0.5	C	0.13	0.14
<i>CXCL1</i>	rs3117604	C	0.43	0.95	C	0.41	0.88
<i>CXCL2</i>	rs3806792	NA	NA	NA	T	0.44	0.5
<i>HSPA1B</i>	rs2763979	T	0.37	0.16	T	0.38	0.13
<i>TNFα-308G/A</i>	rs1800629	A	0.11	0.37	A	0.15	0.36

Abbreviations: MA, minor allele; MAF, minor allele frequency; HWE, Hardy–Weinberg Equilibrium; NA, not applicable, non-polymorphic marker.

Table 3 Genetic Association Of The Selected SNPs With Alopecia Areata Susceptibility

Gene	SNP	Allele/Genotype	Control (n= 150)	Case (n= 152)	Cases Vs Control	
					Chi-sq	P-value
TAP 1	rs2071480	A	(144) 49%	(158) 53%	0.962	0.326
		C	(152) 51%	(142) 47%		
		A/A	(40) 27%	(42) 28%	2.0	0.36
	rs1135216	A/C	(64) 43%	(74) 49%		
		C/C	(44) 3%	(34) 23%		
		T	(256) 86%	(270) 89%	0.753	0.385
rs1057141	C	(40) 14%	(34) 11%			
	C/C	(4) 3%	(3) 2%	0.70	0.70	
	T/C	(32) 22%	(28) 18%			
	T/T	(112) 76%	(121) 80%			
	T	(254) 86%	(264) 87%	0.135	0.71	
	C	(42) 14%	(40) 13%			
CXCL1	rs3117604	C/C	(4) 3%	(5) 3%	0.524	0.77
		T/C	(34) 23%	(30) 20%		
		T/T	(110) 74%	(117) 77%		
	CXCL2	T	(170) 57%	(178) 59%	0.14	0.71
		C	(126) 43%	(124) 41%		
		C/C	(27) 18%	(25) 17%	0.16	0.92
HSPA1B	rs2763979	T/C	(72) 49%	(74) 49%		
		T/T	(49) 33%	(52) 34%		
		T	(136) 5%	(160) 56%	2.52	0.11
	rs3806792	C	(138) 5%	(124) 44%		
		C/C	(35) 26%	(47) 33%	2.4	0.298
		C/T	(66) 48%	(66) 46%		
TNF α -308G/A	rs1800629	T/T	(36) 26%	(29) 2%		
		C	(186) 63%	(187) 62%	0.196	0.658
		T	(108) 37%	(117) 38%		
	rs1800629	C/C	(63) 43%	(62) 41%	0.17	0.92
		C/T	(60) 41%	(63) 41%		
		T/T	(24) 16%	(27) 18%		
rs1800629	G	(261) 89%	(257) 85%	3.07	0.079	
	A	(31) 11%	(47) 15%			
	A/A	(0) 0%	(5) 3%	5.5	0.022	
	G/A	(31) 21%	(37) 24%			
	G/G	(115) 79%	(110) 72%			

Note: $P < 0.05$ is considered to be statistically significant.

Table 3) by using chi-square test in order to determine if there is a significant linkage between the genotypic and allelic frequencies with AA susceptibility.

The genotype frequencies of rs1800629 G/A and A/A within the *TNF α* gene in chromosome 6 show the highest association with AA susceptibility in the overall estimated effects ($P = 0.022$ and 0.0079 , respectively). However, no significant differences were detected in the allele frequencies between AA cases and controls ($P = 0.079$ for allele

frequency) as shown in Table 4. The heterozygous and rare homozygous (G/A and A/A) genotypes were found to be more frequent among alopecia cases (24% and 3%, respectively) than controls (21% and 0%, respectively). On the other hand, the common homozygous genotype (G/G) showed less frequency among cases (72%) in comparison to the controls (79%) (Table 3). Figure 1 shows a scatter plot of the rs1800629 within the *TNF α* from sequenom data whereas each point representing the measurement for a single

Table 4 Genetic Association Of rs1800629 Within The *TNF α* Gene Between Alopecia Areata And Healthy Individuals Using Different Genetic Models

SNP ID	Model	OR	95% CI	P-value
rs1800629	Codominant (G/A)	1.23	0.71–2.15	0.022
	Dominant (G/G)	1.41	0.82–2.42	0.21
	Recessive (A/A)	NA	NA	0.0079
	Log additive ()	1.55	0.94–2.54	0.082

Note: $P < 0.05$ is considered to be statistically significant.

individual. The points were colored according to the genotype calls. Genotypes of the 7 SNPs were highly accurate with an average success rate of 98% and the discrepancy average (\pm SD) rate across the 7 loci was only 0.02 ± 0.0003 in the whole cohort (302 subjects). Allele frequencies and genotypes of the studied SNPs within *TAP1*, *CXCL1*, *CXCL2*, and *HSPA1B* genes revealed no significant differences between AA patients and the healthy individuals (Table 2).

Genetic association analysis of the rs1800629 in *TNF α* gene was investigated using different genetic statistical methods: dominant, additive and recessive genetic models for this associated SNP. The co-dominant form G/A and the rare homozygous A/A were found to be significantly associated depending on the chi-square results. However, no genetic association was found within the common homozygous G/G ($P > 0.05$) (Table 4). For the 3 haplotypes of the *TAP1* gene, there was no significant association with AA with $P > 0.05$ (Table 5).

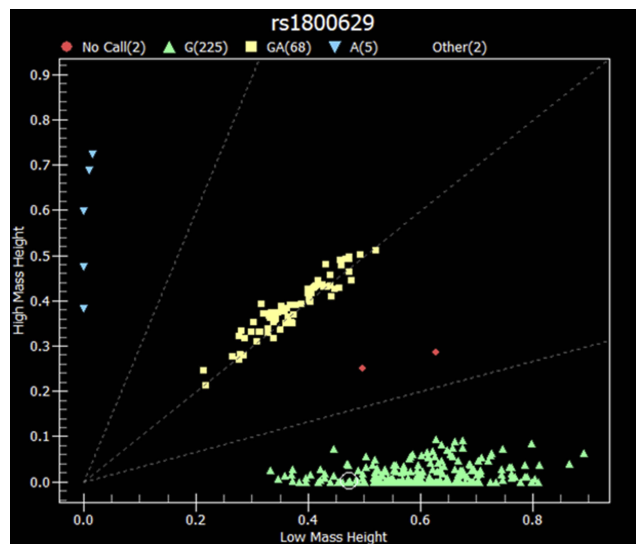


Figure 1 Representative scatter plot of rs1800629 SNP within the *TNF α* gene from sequenom data. The X and Y denote the mass height measurement for the two alleles. Green color (GG) genotype; yellow color (GA) genotype, blue color (AA) genotype, and red color denotes no call ($n = 302$).

Table 5 Genetic Haplotype Frequencies Of *TAP1* Gene (rs1057141, rs1135216 And rs2071480) Associated With Alopecia Areata

Genetic Haplotype	Total	Control Status	Patient Status	P-value
TTC	0.4479	0.4675	0.4283	NA
TTA	0.4137	0.3872	0.4401	0.28
CCA	0.0761	0.0857	0.0666	0.63
CCC	0.0455	0.046	0.0452	0.93
CTA	0.015	0.0102	0.0197	0.35
TCA	0.0017	0.0034	NA	NA

Note: $P < 0.05$ is considered to be statistically significant.

Abbreviation: NA, not applicable.

Discussion

Alopecia areata is thought to be a tissue-specific autoimmune disease directed against the hair follicle and might be associated with other autoimmune diseases.¹⁵ Previous studies showed that MHC genes of chromosome 6 which encodes the human leukocyte antigens (HLAs) are major determining loci for T-cell-mediated diseases including AA.⁶ A number of organ-specific autoimmune diseases have been identified, including diabetes, autoimmune thyroiditis, psoriasis, inflammatory bowel diseases, rheumatoid arthritis, and systemic lupus to be associated with specific alleles of HLA or MHC.⁴ However, HLA contribution alone could not be enough for the explanation of the entire genetic basis of AA.¹⁶ Several studies found that AA has a polygenic origin where multiple genes intertwine causing a greater susceptibility to develop the disease.¹⁷ A recent genome-wide association (GWAS) study revealed the association of HLA in addition to new loci outside the MHC including ACOXL/BCL2L11 and GARP with a nominal association of SH2B3(LNK)/ATXN2.¹⁸

So far, *IFN- γ* , interleukins and *TNF- α* are cytokines with a major role in the pathogenesis of autoimmune diseases including AA. Previous studies published since the 1980s and early 1990s suggested cytokine and chemokine role in autoimmunity.^{19–21} A present study indicates a crucial role of the cytokine as an inducer of hair loss with AA.²² *TNF α* is known to be a risk factor in a variety of inflammatory pathologies.²³ Our data show an association of the *TNF α* with AA that goes in agreement with the study by Gregoriou et al who found that patients with multiple lesions contain extremely high levels of B-cell-activating factor that belongs to the TNF family.¹³ This indicates an increase in the *TNF α* levels in patients with AA which has been confirmed in the present study. However, another

study performed by Galbraith and Pandey in USA suggested no significant difference in the distribution of *TNF- α* G/A phenotypes between patient groups.²⁴ Their results provide direct evidence of genetic heterogeneity between the two forms of AA. They also suggested that the *TNF- α* gene or a closely linked locus within the MHC might play a role in the pathogenesis of the patchy form of the disease.²⁴

In this study, an association between rs1800629 of the *TNF- α -308G/A* and AA was confirmed ($P= 0.022$) and found to be consistent with the results of Gregoriou et al.¹³ It is also consistent with another study performed by Cristina et al in Mexico which suggested a credible association between the presence of *TNF- α -308G/A* polymorphism and an increased susceptibility for the development of patchy AA.²³ This overproduction of *TNF α* might lead to a higher risk of facilitating an autoimmune response against the hair follicle. In our data, the variant's genotype association between AA cases and control was confirmed using different genetic models in order to analyze the genetic association. The association was found in the heterozygous G/A and rare homozygous A/A versus common homozygous G/G. These findings provide evidence of genetic variants in *TNF α* gene with AA development in the Jordanian population. Upon a recent screening, the EBI-GWAS catalog stated that rs1800629 variant is located in the MHC region and there is an extensive linkage disequilibrium (LD) in the HLA region where the association signal of rs1800629 might be due to LD with neighboring variants associated with AA. EBI-GWAS catalog indicates that rs9275524-C is close to HLA-DQB1 and it is in LD with rs1800629 and associated with AA.²⁵ Therefore, the weak association signal of rs1800629 in this study is likely due to LD and cannot be used to implicate *TNF α* as the causal gene without considering other correlated variants.

Mast cells and macrophages both conduce in the early stages of recruiting neutrophils into tissues. CXCL1 and CXCL2 are neutrophil chemoattractants produced in response to the neutrophil attack by mast cells and macrophages.²⁶ Chemokines play important roles in the development, homeostasis, and function of the immune system as well as angiogenesis.²⁷ For that reason, this study focused on the role of these genes in the development of AA. A study performed by Kim et al on the Korean population confirmed that genetic sequencing of the rs3117604 and rs3806792 in *CXCL1* and *CXCL2* gene, respectively, showed a significant correlation between the

two SNPs and the development of AA.²⁷ It also confirmed that T/T and C/C haplotypes were significantly associated with an increased risk of AA. In contrast, this study revealed no significant association of the *CXCL1* and *CXCL2* with AA susceptibility. On the other hand, none of the polymorphisms differed significantly for allele or genotype frequencies among cases and controls.

HSPA1B plays a major role in immunity mainly in MHC class III, and different levels of HSPA1B expression correspond to hair loss and immunologic reactions in anagen (i.e. first stage in hair development) hair follicles, which are common features in AA histopathology.¹¹ In a previous study presented by Seok et al in Korea, rs6457452 was found to be associated with the onset of AA. In their analysis of clinical features obtained for AA, rs6457452 was only weakly related to the age of onset, and rs2763979 was only weakly related to the type of AA lesion.¹¹ In conclusion, they suggested that rs6457452 might be associated with the onset of AA, and the T allele might grant the reduced susceptibility to AA in the Korean population. Contrary to our results that revealed no association of the rs276379 in the Jordanian population.

Investigation of polymorphisms in *TAP1* identified an association with several autoimmune diseases and AA is one of them.²⁸ The study of Kim et al was one of the first documented studies in comparing genotypic and allelic frequencies of *TAP1* in patients with AA.²⁸ In their study, Korean patients were tested for three selected SNPs (rs2071480, rs1135216, and rs1057141), whereas results suggested an association of a promoter SNP (rs2071480) in the heterozygous G/T co-dominant. In addition, the homozygous T/T genotype showed a significant association with an increased risk of AA. In contrast, none of the three aforementioned SNPs were associated with AA in this study.

Differences in ethnicity groups, type of AA, linkage equilibrium and disease duration could be a probable clarification for such contradictory results reported in the literature. However, most of the studies in Arab populations were restricted by being of a retrospective design since most of them have short follow-up periods. The future direction toward inclusion of a larger cohort and additional genes and loci based on the newly GWAS studies conducted on the association of the AA.

Conclusion

This study further supports the polygenic model of AA. *TNF α* seems to have an essential role in AA pathogenesis

with a statistically significant association. This finding indicates the importance of potential researches for sequencing of the HLA region where *TNF α -308G/A* could be of linkage equilibrium with other variants as previously performed in other ethnic groups. In the current study, no significant role was identified for genetic variants of chemokines (*CXCL1* and *CXCL2*), heat shock protein (*HSPA1B*), and ATP-binding cassette (*TAP1*) to influence the development of AA in the Jordanian population since no genetic association was found with AA susceptibility as well as for the *TAP1* haplotypes. Furthermore, no correlation was found between the tested genetic changes and the clinical phenotypes.

Ethics Approval And Informed Consent

All procedures contributing to this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and the Declaration of Helsinki with an IRB no. (48/2017).

Data Availability

The datasets generated and analyzed during the current study are not publicly available. The consent from participants did not cover data sharing but are available from the corresponding author on reasonable request.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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