RESEARCH ARTICLE

Genetic analysis of interleukin 18 gene polymorphisms in alopecia areata

Sumeyya Deniz Celik | Omer Ates

Department of Medical Biology, Medical Faculty, Gaziosmanpasa University, Tokat, Turkey

Correspondence

Sumeyya Deniz Celik, Gaziosmanpasa University, Medical Faculty, Department of Medicine Biology, Tokat, Turkey.
Email: sumeyyadenizcelik@gmail.com

Background: Alopecia areata (AA), which appears as nonscarring hair shedding on any hair-bearing area, is a common organ-specific autoimmune condition. Cytokines have important roles in the development of AA. Interleukin (IL) 18 is a significant proinflammatory cytokine that was found higher in the patients with AA. We aimed to investigate whether the IL-18 (rs187238 and rs1946518) single nucleotide polymorphisms (SNPs) may be associated with AA and/or clinical outcome of patients with AA in Turkish population.

Methods: Genotyping of rs187238 and rs1946518 SNPs were detected using sequence-specific primer-polymerase chain reaction (SSP-PCR) method in 200 patients with AA and 200 control subjects.

Results: The genotype distribution of rs1946518 (–607C>A) SNP was found to be statistically significantly different among patients with AA and controls ($P = .0008$). Distribution of CC+CA genotypes and frequency of –607/allele C of rs1946518 SNP were higher in patients with AA ($P = .001$, $P = .001$, respectively). The genotype distribution of rs187238 (–137G>C) SNP was found to be statistically significantly different among patients with AA and control subjects ($P = .0014$). Distribution of GG genotype and frequency of –137/allele G of rs187238 SNP were higher in patients with AA ($P = .0003$, $P = .001$, respectively).

Conclusion: The rs1946518 (–607C>A) and rs187238 (–137G>C) polymorphisms were found associated with alopecia areata disease. The study suggests that IL-18 rs187238 and rs1946518 SNPs may be the cause of the AA susceptibility.

KEYWORDS

alopecia areata, autoimmunity, interleukin 18, polymorphism

1 | INTRODUCTION

Alopecia areata (AA), which is a common T lymphocyte cell-mediated, organ-specific autoimmune condition, is clinically described by different size and forms of nonscarring hair loss lesions on any hair-bearing area.^{1,2} The prevalence of the disease is between 1% and 2% worldwide, and it takes place at any age from birth to late life.³ AA can suddenly occur in any hair-bearing area, and it can be classified considering the degree and pattern of the hair shedding.⁴ Etiopathogenesis of the AA is not still entirely understood, but some studies suggest that hair follicles are distorted by T lymphocyte cells.^{5–7} In these immunological

responses, cytokines, which are produced from T lymphocytes, are considered as important players in autoimmune inflammation.⁵

Interleukin (IL) 18, which stimulates to produce interferon (IFN)- γ , is an important proinflammatory cytokine, produced in various immune and nonimmune cells.^{8–10} IL-18 induces IFN- γ production from natural killer (NK) and Th1 cells, to activate Th1 cell proliferation and to increase the cytotoxic effect of NK cell.¹¹ IL-18 has pathogenic roles in autoimmune diseases so that the cytokine production is a major incident of the innate immune response. In experimental and clinical studies, higher levels of IL-18 constantly associated with the intensity of autoimmune diseases.¹²

Two functional gene polymorphisms (−607C>A and −137 G>C) replace transcription factor binding sites and expression level of IL-18.¹³ Plenty of studies have examined the association among IL-18 SNPs and inflammatory and/or autoimmune conditions such as systemic lupus erythematosus and rheumatoid arthritis.^{14–16} Only one study examined the roles of IL-18 SNPs in AA disease, and they reported that the −137G>C and Ser35Ser polymorphisms were associated with AA. IL-18, which stimulates IFN- γ , is a major cytokine in the Th1 response and affects the development of autoimmune, inflammatory, and infectious diseases. For those reasons, the study hypothesized that rs1946518 and rs187238 SNPs may be associated with etiopathogenesis of AA.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study included 200 patients with AA (mean age $\pm$ SD: 36.62 $\pm$ 9.6 years) and 200 control subjects (mean age $\pm$ SD: 37.38 $\pm$ 11.31). In patients with AA, 123 were males and 77 were females, and among the control subjects, 120 were males and 80 were females. The patients with AA and control subjects were collected from Dermatology Clinic of Gaziosmanpasa University, Turkey. The criteria for the identification of AA were regarded standard criteria.¹⁷ Clinical data were acquired from 200 patients with AA. The control subjects consist of patients with no AA history, who were referred to dermatology clinic for other reasons. Our study protocol was approved by the Gaziosmanpasa University hospital's ethics committee. Written informed consent was obtained from both patients with AA and control subjects, who were also acknowledged about the blood sampling for rs187238 and rs1946518 SNPs genotype analyses.

2.2 | DNA isolation

Peripheral blood samples from both patients with AA and control subjects were collected, and genomic DNAs (gDNA) were purified using a GeneJET gDNA purification kit (Thermo Scientific™, USA).

2.3 | Genotyping

Single nucleotide polymorphism (SNP) genotyping was examined using sequence-specific primer-polymerase chain reaction (SSP-PCR) method, according to the previous reports.¹⁸ For rs 1946518 and rs187238 polymorphisms, the PCR primers and PCR product lengths are shown in Table 1. For rs 1946518, the PCR mixtures were prepared in 25 μ L of a solution containing 2 μ L of gDNA, 0.25 μ L of 10 mmol of dNTP, 1.5 μ L of 25 mmol/L MgCl₂, 2.5 μ L of 10 $\times$ buffer, 0.5 U of *Taq* polymerase, 0.5 μ L of one sequence-specific forward primer (for allele C or -A), 0.5 μ L of the common reverse primer, and 0.15 μ L of internal positive control primer.

For rs187238, the PCR mixtures were prepared in 25 μ L of a solution containing 2 μ L of gDNA, 0.25 μ L of 10 mmol of dNTP, 1.5 μ L of 25 mmol/L MgCl₂, 2.5 μ L of 10 $\times$ buffer, 0.5 U of *Taq* polymerase, 0.5 μ L of reverse primer and sequence-specific forward primer (for allele G or allele C), and 0.5 μ L of the internal positive control primer. Agarose gel electrophoresis is used to visualize the PCR products.

2.4 | Statistical analysis

All statistical data were analyzed by OpenEpi Info software version 3.01 and SPSS 20. Our results are reported as mean $\pm$ standard deviation. The relationships among IL-18 SNPs and clinical and demographics characteristics of AA were examined by chi-square test. The Hardy-Weinberg equilibrium (HWE) was analyzed by chi-square test.

TABLE 1 Polymorphisms, the PCR primers, PCR product lengths, and PCR program

Polymorphisms	Primer sequence	PCR Product Lengths	PCR program	
IL-18 rs187238 (−137G>C)	R: 5′-AGGAGGGCAAAATGCACTGG-3′		94°C	2 min
	F G: 5′-CCCCAACTTTTACGGAAGAAAAG-3′	261 bp	94°C	20 s
	F C: 5′-CCCCAACTTTTACGGAAGAAAAC-3′	261 bp	68°C	60 s
	CTRL: 5′-CCAATAGGACTGATTATTCGCA-3′	446 bp	72°C	40 s
			94°C	20 s
			62°C	20 s
IL-18 rs1946518 (−607C>A)			72°C	40 s
	R: 5′-TAACCTCATTGAGGACTTCC-3′		94°C	2 min
	F C: 5′-GTTGCAGAAAGTGTAATAATTATTAC-3′	196 bp	94°C	20 s
	F A: 5′-GTTGCAGAAAGTGTAATAATTATTAA-3	196 bp	64°C	40 s
	CTRL: 5′-CTTGTCTATCATTCCAGGAA-3′	301 bp	72°C	40 s
			94°C	20 s
			57°C	40 s
			72°C	40 s

IL-18, interleukin 18, R, common reverse primer, F, forward primer, CTRL, internal positive amplification control, bp, base pair.

TABLE 2 The demographical characteristics of patients with AA and control subjects

	Patients with AA n = 200	Control subjects n = 200	P value
Gender			
Female	77 (38)	80 (40)	.84
Male	123 (62)	120 (60)	
Age (y) mean $\pm$ SD	36.62 $\pm$ 9.6	37.38 $\pm$ 11.31	.483

Data were examined by analysis of variance and chi-square test, and mean $\pm$ standard deviation values are shown for age.

AA, alopecia areata; SD, standard deviation.

Categorical variables were evaluated appropriately using the chi-square test. Odds ratios (ORs) with 95% confidence intervals (CIs) were used, and *P* values less than .05 were regarded statistically significant and were two-tailed.

3 | RESULTS

Genotype distribution and allelic frequencies of rs187238 and rs1946518 SNPs located in the promoter site of IL-18 gene, respectively, were examined in patients with AA and control group. The demographical characteristics of patients with AA and control subjects in our study groups are summarized in Table 2. According

to gender distribution, there were no significant differences among patients and control subjects (*P* = .84). There was no significant mean age differences among patients with AA and control subjects (*P* = .483). Baseline clinical and demographical characteristics of patients with AA are demonstrated in Table 3. No difference was shown among clinical and demographical characteristics of patients with AA and rs187238 and rs1946518 polymorphisms. Genotype distribution for rs187238 and rs1946518 promoter polymorphisms fits the HWE. The genotypic distributions and allelic frequencies of rs187238 and rs1946518 polymorphisms in patients with AA and control group are shown in Table 4. For rs187238 polymorphism, the distribution of the wild G/G genotype was found significantly higher in patients with AA (64.5%) than in control group (46.5%). The distribution of the G/C genotype was higher in controls (39.5%) than in patients (25.5%), and distribution of the C/C genotype was found significantly lower in patients with AA (10%) than in controls (14%). Genotype distribution of rs 187238 SNP was significantly different among patients with AA and controls (*P* = .0014). The frequency of the allele G was significantly higher in patients (77.25%) than in control subjects (66.25%), while an important increase in the frequency of the allele C was shown in control subjects (33.75%) when compared to patients (22.75%) (*P* = .001, OR = 1.73, 95% CI = 1.27-2.37). The distribution of GC+CC (for the dominant model: GC+CC vs GG) was significantly higher among controls than patients (53.5% vs 35.5%, respectively, *P* = .0003, OR = 2.08, 95% CI = 1.39-3.12).

TABLE 3 Baseline clinical and demographical characteristics of the 200 patients with AA

Characteristic	Study group	P	
		IL-18 (rs1946518, -607C>A)	IL-18 (rs187238-137G>C)
Gender, no. of male/female (%)	77/123 (38/62)	.372	.523
Age (y)	36.62 $\pm$ 9.6	.844	.237
Disease duration (mo)	21.80 $\pm$ 44.39	.310	.764
Number of attacks	1.23 $\pm$ 3.434	.483	.521
Family history positive, n (%)	6 (3.0)	1.000	.804
Focal infection positive, n (%)	18 (9.0)	.554	.870
Nail dystrophy positive, n (%)	60 (30.0)	.523	.784
Stress positive, n (%)	110 (55.0)	.328	.362
Alopecia severity, n (%)			
<50%	193 (96.5)	.901	.812
>50%	7 (3.5)		
Alopecia localization, n (%)			
Eyebrow	7 (3.5)	.644	.591
Scalp	142 (71)		
Beard/mustache	47 (23.5)		
Eyelash	4 (2)		

Data were examined by analysis of variance and chi-square test, and mean $\pm$ standard deviation values are viewed for age and age at disease onset.

TABLE 4 Genotype distributions and allele frequencies of IL-18 rs1946518 and rs187238 polymorphisms in patients with AA and control subjects

Polymorphism	Patients with AA n = 200 (%)	Control subjects n = 200 (%)	P	OR (95% CI)
IL-18 rs1946518 (-607C>A)				
Genotypes				
CC	75 (%37.5)	50 (%25)	.0008	
CA	108 (%54)	110 (%55)		
AA	17 (%8.5)	40 (%20)		
CC: CA+AA	75 (%37.5):125 (%62.5)	50 (%25):150 (%75)	.007	1.79 (1.17-2.77)
CC+CA: AA	183 (%91.5): 17 (%8.5)	160 (%80):40 (%20)	.001	2.68 (1.47-5.02)
Alleles				
C	258 (%64.5)	210 (%52.5)	.001	1.64 (1.24-2.18)
A	142 (%35.5)	190 (%47.5)		
IL-18 rs187238 (137G>C)				
Genotypes				
GG	129 (%64.5)	93 (%46.5)	.0014	
GC	51 (%25.5)	79 (%39.5)		
CC	20 (%10)	28 (%14)		
GG: GC+CC	129 (%64.5):71 (%35.5)	93 (%46.5):107 (%53.5)	.0003	2.08 (1.39-3.12)
GG+GC: CC	180 (%90):20 (%10)	172 (%86):28 (%14)	.22	
Alleles				
G	309 (%77.25)	265 (%66.25)	.001	1.73 (1.27-2.37)
C	91 (%22.75)	135 (%33.75)		

Statistically significant results are shown in boldface.

AA, alopecia areata; OR, odds ratio; CI, confidence intervals.

For rs1946518 polymorphism, the distribution of the wild C/C genotype was found significantly higher in patients with AA (37.5%) than in control subjects (25%). The distribution of the C/A genotype was found as higher in controls (55%) than in patients with AA (54%), and distribution of the A/A genotype was found significantly lower in patients with AA (8.5%) than in controls (%20). Genotype distribution of rs1946518 SNP was significantly different among patients and controls ($P = .0008$). The frequency of the -607/allele C was significantly higher in patients with AA (64.5%) than in control subjects (52.5%) while an important increase in the frequency of the A allele was viewed in control subjects (47.5%) when compared to patients with AA (35.5%) ($P = .001$, OR = 1.64, 95%CI = 1.24-2.18). While the distribution of CA+AA (for the dominant model: CA+AA vs CC) was significantly higher among controls than patients (75% vs 62.5%, respectively, $P = .007$, OR = 1.79, 95% CI = 1.17-2.77), the frequency of CC+CA (for the recessive model: CC+CA vs AA) was significantly higher among patients than controls (91.5% vs 8.5%, respectively, $P = .001$, OR = 2.68, 95% CI = 1.47-5.02).

4 | DISCUSSION

In AA, the molecular mechanisms that give rise to hair shedding are not still clear.⁴ On the other hand, genetic factors have significant

roles in the etiopathogenesis of AA and certain gene alleles have been associated with the disease.^{19,20} Petukhova et al found eight different genomic regions associated with susceptibility to AA in genome-wide association study. They suggested that most of these regions have an important role in T-cell-mediated pathways.²¹

IL-18 is a considerable proinflammatory cytokine for T-cell-mediated autoimmunity as it can increase the production of Th1-Th2 cytokines from stimulating T lymphocytes.²²

Throughout the development of AA, class I-II MHCs are highly expressed by the follicular epithelium and some studies also show that MHC class I-II gene expressions are induced by IFN- γ on hair follicle epithelium.^{23,24} Gilhar et al have reported accelerated development of AA by intradermal injection of IFN- γ to mouse model. In addition, Freyschmidt-Paul et al²⁴ suggest that IFN- γ -mediated Th1 activation is necessary for stimulating an AA-specific immune response. These findings show that IL-18 may be related to the process for that induces IFN- γ production from NK and Th1 cells. Stress is considered as a trigger for initiation of AA, and it is also showed that adrenocorticotrophic hormone (ACTH), which is a stress hormone, is increased in AA.²⁰ Some studies showed that IL-18 cytokine may be associated with stress. In addition, Sekiyama et al²⁵ suggested that stress induces ACTH which induces transformation from IL-18 precursor proteins to mature IL-18 via caspase-1 in the adrenal cortex. Thus, proinflammatory IL-18 cytokine levels are elevated in the adrenal gland and plasma. Wang et al²⁶

investigated the development of heart disease in AA-affected C3H/HeJ mice which are exposed to ACTH. They found that IL-18, IL-18 binding protein, and IL-18 receptor-1 increased in AA-affected C3H/HeJ mice.

IL-18 has an important act in stimulation of Th1-2 cytokines, and also it suggested that IL-18 promoter polymorphisms can impress the balance among Th1 and Th2 cytokine responses. Giedraitis et al¹⁸ found that -607/allele C or -137/allele G showed higher transcription activity of IL-18. Individuals, which carry alleles with higher activity (-607/C or -137/G), have higher resistance against infections while they have more susceptibility to autoimmune diseases than individuals have lower activity alleles (-607/A or -137/C).¹⁸ According to our results, genotype distribution and allele frequency of rs187238 SNP were found to be statistically different among patient and control groups ($P = .0014$ and $P = .001$, respectively). Genotype distribution and allele frequency of rs1946518 polymorphism were also found to be significantly different among patient and control groups ($P = .0008$ and $P = .001$, respectively). The distributions of -607/CC and -137GG genotypes showed a significant increase, while genotypes -607/AA and -137/CC showed a significant decrease in patients with AA as compared to the control subjects.

IL-18 polymorphism was found to be associated with systemic lupus erythematosus (SLE), which is an immune-mediated disease.¹⁶ Fouad and others investigated IL-18 SNPs (rs1946518, rs187238) in patients with SLE, and they found that the distribution of -607/CC genotype was significantly higher, while genotype -607/CA was significantly lower in patients with SLE than control individuals. In addition to plasma, IL-18 level was found to be significantly elevated in patients with SLE with genotype CC at -607.¹⁴ Kim et al¹⁰ investigated whether IL-18 -137G>C and exonic Ser35Ser polymorphisms are associated with AA in Korean population. This was the first study to analyze whether or not IL-18 polymorphism is associated with AA, and it also showed that the -137G>C and Ser35Ser polymorphisms were associated with AA disease. Also, they found that the frequency of the allele G of -137G>C polymorphism in the AA group was found higher than the control group ($P = .023$). There was a difference in our study compared to the study of Kim and others. Our study firstly showed that there was an association among AA disease and IL-18 rs1946518 (-607C>A) polymorphism. Lee et al also found that serum IL-18 cytokine levels in patients with extensive AA were significantly elevated than in controls.²⁷

Finally, our study demonstrated that IL-18 SNPs, the -137/G and -607/C allelic variants which relate to the increased production of IL-18, may be a significant genetic factor for AA. The status explains the increased level of serum IL-18 in patients with AA. IL-18 SNP should be examined in large cohort, which contains different ethnic groups. Furthermore, IL-18, its receptors, and inhibitors will be more important to understand the pathogenesis of AA.

AUTHORS' CONTRIBUTIONS

SDC and OA conceived the study; SDC and OA collected and processed the data; SDC and OA participated in interpretation; SDC and

OA performed genetic analysis; SDC and OA reviewed the literature; SDC wrote the article; OA critically reviewed the article. The authors thank Goknur Kalkan for the critical review of the article and the kind assistance in obtaining the patient's samples.

ORCID

Sumeyya Deniz Celik <http://orcid.org/0000-0001-9957-5485>

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