Research Article

Identification of Single Nucleotide Polymorphisms as Markers of Genetic Susceptibility for Alopecia Areata Disease Risk

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Abstract | **Background**: Alopecia areata (AA) is an autoimmune disease, leading to disfiguring hair loss that susceptibility loci and the genetic basis of AA have been largely unknown. **Objective**: The aim of this study was the scrutiny the susceptible genes of Alopecia areata amongst patients and healthy adult in Iranian populations. **Methods**: four variants polymorphisms (rs1701704, rs10760706, rs9275572, rs694739) were studied by Tetra Arms PCR, Sequencing methods in 200 Iranian healthy adult blood donors and 200 patients with Alopecia Areata (AA). **Results**: Results were showed that 4 SNPs had P-values <0.05 for association with Alopecia areata. 3 of 4 SNPs, was demonstrated significant association in analyses 100 AT/AU cases versus 100 AA, which is localised in *IKZF4, STX17,* PRDX5, *HLA-DQB1* (rs1701704, rs10760706, rs694739 and rs9275572 respectively). **Conclusions**: In this study, 3 of 4 SNP-associated loci were associated significantly with association with the development of Alopecia areata. In another word, the presence of them may be a contributing factor for prognosis of the development of the disease to Totalis and Universalis.

Keywords | Alopecia Areata (AA), Alopecia Universalis (AU), Alopecia Totalis (AT), autoimmune disease

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INTRODUCTION

Alopecia areata (AA) is a common autoimmune disease with a variable in humanity that leading to non-scarring hair loss (Safavi et al., 1995; Petukhova et al., 2010; Pratt et al., 2017; Sadeghi et al., 2015; Behrangi et al., 2017). AA is a multifactorial disease which needs several environmental and genetic factors to immune privilege of the hair follicle collapses, and subsequent autoimmune attack will occur (Petukhova et al., 2010; Pratt et al., 2017; Behrangi et al., 2017). The disease can take many forms ranging from a loss in well-defined patches (AA), or diffuse hair loss in the form of total loss of scalp hair called alopecia totalis (AT), or loss of entire scalp and body hair called alopecia universalis (AU), which can affect all hair-bearing sites. Patchy alopecia affecting the scalp is the most common type (Pratt et al., 2017; Alsantali, 2011; Sadeghi et al., 2015). Typically, AA could relapse or remit and also that can be persistent – especially when hair loss is extensive (Pratt et al., 2017; Behrangi et al., 2017).

However genetic basis of AA has been largely unknown,

there are several evidences supporting them, including the observed heritability in first-degree relatives, twin studies and, most recently, from family-based linkage studies (Petukhova et al., 2010; McDonagh and Tazi-Ahnini, 2002; Van der Steen et al., 1992; Jackow et al., 1998; Martinez-Mir et al., 2007). Genetic research in patients and mouse models showed that several genetic susceptibility loci are associated with signalling pathways that are pivotal to hair follicle cycling (Pratt et al., 2017; Malani, 2014; Sundberg et al., 1994). Unfortunately, these studies were limited by small sample sizes and preselection of candidate genes. Recent advances in comprehension of the molecular mechanisms of AA by application of genome-wide association studies (GWAS) that have identified candidate genes associated with susceptibility to alopecia areata have revealed new treatments and the possibility of remission in the near future (Petukhova et al., 2010; Jabbari et al., 2016; Gip et al., 1969). Therefore, all these techniques and affiliated observations are responsible for that Alopecia areata has been now firmly known as a complex, polygenic, immune-mediated disease (Jabbari et al., 2016).

Single nucleotide polymorphisms (SNPs) is a common type of variation in the DNA sequence occurring in greater than one percent of the population. Individuals may be homozygous or heterozygous for an SNP at a specific site of the genome due to They may inherit them from their parents. These SNPs may lead to different actions base on their location on the genome such as they are located within the regulatory regions of the genes which may influence the expression of the gene, or they are located within the exons or exon-intron boundaries which may modify the protein function or the splicing sites, respectively (Zienoldiny and Skaug, 2012).

A genome-wide association study identified 139 single nucleotide polymorphisms that are significantly associated with AA ($P \leq 5 \times 10^{-7}$) (Petukhova et al., 2010). It showed an association with genomic regions including several genes involving in autophagosome such as *STX17* or genes participating in the cellular response to oxidative stress such as PRDX5 or some genes controlling the activation and proliferation of regulatory T cells (Treg cells), cytotoxic T lymphocyte-associated antigen 4 (*CTLA4*), interleukin (IL)-2/IL-21, IL-2 receptor A (*IL-2RA*; CD25) , *IL18* , Eos (also known as Ikaros family zinc finger 4; *IKZF4*), as well as the human leukocyte antigen (*HLA*) region. (Petukhova et al., 2010; Song et al., 2013; Zhang et al., 2005; Rosengren Pielberg et al., 2008; Akar et al., 2002; Wang et al., 2002; Karasawa et al., 2005).

In present study was analysed the genetic risk factors contribute to AA. Odd ratio of SNPs (rs1701704, rs10760706, rs9275572, rs694739) presence on gene was investigated amongst patients with alopecia and healthy group.

MATERIAL AND METHODS

Subject Selection and Sampling

Subjects were included 200 patients with Alopecia Areata and 200 healthy adult subjects. The age range of patients was 15-40 years old, and control subjects were 40 -50 years old. Healthy adult subjects had not any current infection and history of autoimmune or allergic diseases or D3 vitamin deficiency. In addition, there was no Cousin marriage up to three previous generations (Table 1). K2 EDTA tube (VACUETTE® EDTA) was used to collect 2cc of the acquired peripheral blood sample. All participants have signed a written informed consent.

Table 1: Demographic and clinical characteristics of alopecia areata patients and the control subjects

	Patients		Control
	AA	*AT/AU*	
Number of subjects	100	100	200
Male/female	117/83		113/87
Age (mean±SD)	27.5±12.5		45±5
First onset age			
<30 years	189		
≥30 years	11		
Family Hx for Alopecia areata			
Presence	52		0
Absence	148		200
Cousin marriage			
Presence	185		0
Absence	15		200
Involvement of nail			
Presence	134		
Absence	66		
D3 vitamin deficiency			
Presence	187		0
Absence	13		200
Infection disease			
Presence	0		0
Absence	200		200
Allergic diseases			
Presence	62		0
Absence	138		200

AA, alopecia areata; N, number of subjects; SD, standard deviation.

DNA Extraction and Primer

Genomic DNA was extracted from blood samples according to protocol DNA extraction of CinnaPureDNA (PR881612-EX6001) kit. Extracted DNA's quality was measured by both 1.5% agarose gel electrophoresis and D-

Table 2: Frequency of SNPs in control and patients with Alopecia areata (AA/AT/AU) (result of analysis I)

SNP	*Genes of Interest*	Type	*Control* n (%)	*Patients* n (%)	*OR (95% CI)*	P
rs1701704	*IKZF4*	C/C	77	54	0.31	0.001
		T/C	15	28	2.28	0.019
		T/T	8	17.5	2.49	0.038
		C	84.5	68.25	0.4	0.007
		T	15.5	31.75	2.5	0.007
rs9275572	*HLA-DQB1*	A/A	81	50.5	0.23	0.0005
		A/G	12.5	21	1.79	0.12
		G/G	6.5	28.5	5.409	0.0005
		A	87.25	61	0.23	0.0005
		G	12.75	39	4.27	0.0005
rs694739	PRDX5	C/C	76	52.5	0.34	0.001
		T/C	16	20	1.31	0.46
		T/T	8	27.5	4.41	0.0005
		C	84	62.5	0.316	0.001
		T	16	37.5	3.16	0.001
rs10760706	*STX17*	T/T	71.5	56.5	0.522	0.028
		T/C	18.5	23.5	1.34	0.39
		C/C	10	20	2.25	0.048
		T	80.75	68.25	0.49	0.035
		C	19.25	31.75	2.006	0.035

The *P* values were calculated from logistic regression analyses adjusting sex and age. Bold numbers mean significance association.
The *P* values were calculated using Bonferroni's correction.
SNP, singe-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 3: Frequency of SNPs in control and patients with Alopecia areata (AA) (result of analysis II)

SNP	*Genes of Interest*	Type	*Control* n *(%)*	*AA* n *(%)*	*OR (95% CI)*	P
rs1701704	*IKZF4*	C/C	77	65	0.55	0.61
		T/C	15	26	1.99	0.054
		T/T	8	9	1.137	0.8
rs9275572	*HLA-DQB1*	A/A	81	65	0.43	0.011
		A/G	12.5	15	1.19	0.66
		G/G	6.5	20	3.35	0.007
rs694739	PRDX5	C/C	76	65	0.58	0.08
		T/C	16	14	0.85	0.69
		T/T	8	21	3.05	0.009
rs10760706	*STX17*	T/T	71.5	65	0.412	0.33
		T/C	18.5	14	0.703	0.35
		C/C	10	21	2.392	0.032

The *P* values were calculated from logistic regression analyses adjusting sex and age. Bold numbers mean significance association.
The *P* values were calculated using Bonferroni's correction.
SNP, singe-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

enovix Nanodrop device (Model Ds-11). Specific primers were designed by using primer3 software and synthesised by SinaColon Company.

Tetra Arms PCR

All thermal-block-based PCR runs were performed in a Biometra TAdvanced thermocycler (Analytik Jena Co, Germany). Table 2 summarises protocol of the PCR reactions. Taq DNA Polymerase Master Mix RED 2X-MgCl2; 1.5mM ampliqon kit (#180301-50) was used. Reaction volumes for the cycler were 25 µL (genomic DNA concentration was 200-250 ng).

Table 4: Frequency of SNPs in patients with Alopecia areata (AA) with patients with AU/AT (result of analysis III)

SNP	*Genes of Interest*	Type	*AA* n *(%)*	*AT/AU* n *(%)*	*OR (95% CI)*	P
rs1701704	*IKZF4*	C/C	65	16	0.406	0.002
		T/C	26	4	1.27	0.43
		T/T	9	80	3.55	0.002
rs9275572	*HLA-DQB1*	A/A	65	27	0.30	0.0005
		A/G	15	27	2.09	0.037
		G/G	20	37	2.34	0.008
rs694739	PRDX5	C/C	65	40	0.35	0.0005
		T/C	14	26	2.15	0.034
		T/T	21	34	1.93	0.04
rs10760706	*STX17*	T/T	48	20	0.49	0.0.15
		T/C	33	35	3.026	0.002
		C/C	19	45	0.88	0.72

The *P* values were calculated from logistic regression analyses adjusting sex and age. Bold numbers mean significance association.
The *P* values were calculated using Bonferroni's correction.
SNP, singe-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

The protocol of PCR amplification for mention polymorphism included of an initial denaturation step at 95 C for 5 min followed by 32 cycles of denaturation at 95 C for 30 s, annealing at 58.2C (rs1701704), 61.5C (rs10760706), 54.7C (rs9275572) and 56.8C(rs694739) for 30s and extension at 72C for 30s. The final extension step was 72 C for 5 min.

PCR products were analysed by electrophoresis in 1.5% agarose gel and Thermo Scientific GeneRuler 100 bp DNA Ladder.

Sequencing

PCR was carried out by using Outer primer (reverse and forward). Then PCR products were sent to SinaColon Company to sequence PCR production in both forward and reverse directions. Sequencing's results were analysed by Finch TV.

Statistics

After data was coded and cleaned, it was analysed by using SPSS version 20. Categorical variables have been summarised by frequency and proportions, and differences amongst the groups were compared using Fisher's exact chi-square analysis. Those variables with 95% CI and p-value less than 0.05 were considered as statistically significant (Table 2, 3 and 4).

RESULTS

In the present study, Tetra Arms PCR method was used to study the polymorphisms. Then DNA sequencing was carried out to ensure the accuracy of previous results. Outcomes of all used techniques were approximately same.

Rs1701704, rs10760706, rs9275572 and rs694739 were studied in 200 healthy adult and 200 patients with Alopecia Areata in Iranian population. There were 230 males (57.5%) (113 healthy adult and 117 AA patients with a frequency 56.5% and 58.5% respectively) and 170 females (42.5%) (87 Healthy adult and 83 AA patients with a frequency 43.5% and 41.5% respectively).

frequency of the homozygous genotype of rs1701704 (T/T), rs9275572 (G/G), rs694739 (T/T) and rs10760706 (C/C) were 8%, 6.5%, 8% and 10% in control group and 17.5%, 28.5%, 27.5% and 20% in patient group respectively (Table 2).

Among the 200 Iranian participants with Alopecia areata (100 patients with Alopecia Areata(AA) and 100 patients with AT/AU), 117 males with a frequency 58.5% and 83 females A 41.5%. They were no significant association.

In the study of two patient groups with AA and AT/AU was stated that 3 SNPs rs1701704 (T/T) (odd ratio = 3.55), rs9275572 (G/G) (odd ratio = 2.34) and rs694739 (T/T) (odd ratio =1.93), a significant association with developing disease ($p<0.05$).

DISCUSSION

Although there is little information about the genetic disease alopecia areata, GWAS study in AA contributed to detecting several suspected genes from the autoimmune aetiology point of view (Petukhova et al., 2010). Identification of susceptible SNPs in the development of the disease may contribute to predict and predict the incidence of disease. In the future, the accurate recognition of these SNPs could lead to comprehending disease developing

mechanism.

In a previous genome-wide association study, Petukhova and et al. (2010) identified a new class of NKG2D ligands in AA (Petukhova et al., 2010). The *ULBP* genes reside on human chromosome 6q25.1. Each of the ULBP genes has been shown to function as an NKG2D-activating ligand (Radosavljevic et al., 2002). Disturbance in the hair follicle microenvironment ostensibly causes the initiation of AA. These results suggested that the autoimmune destruction in AA may be mediated in part by CD8+NKG2D+ cytotoxic T cells, whose activation may be induced by upregulation of ULBP3 in the dermal sheath of the hair follicle (Eagle and Trowsdale, 2007; Eagle et al., 2009). Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4) is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. Mutations in this gene have been associated with insulin-dependent diabetes mellitus, Graves' disease, Hashimoto thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, and other autoimmune diseases. The high expression of *CTLA4* (rs1024161) has been proposed as a significant determinant of their suppressive activity (Monteleone et al., 2009).

In addition, they identified some genetic factors and susceptibility loci may contribute together to induce and promote immune dysregulation in pathogenesis for AA such as *IL2/IL21* (rs7682241), *IL18* (rs187238 and rs549908), *IKZF4* (rs1701704) (Pan et al., 2009), *HLA-DQB1* (rs9275572), *STX17* (rs10760706) and PRDX5 (rs694739) (Zhang et al., 2005; Rosengren Pielberg et al., 2008; Petukhova et al., 2010).

In present study was analysed the risk factor for the development of AA (rs1701704, rs10760706, rs9275572, rs694739) in 200 patients with Alopecia areata and 200 healthy subjects. Odd ratio of SNPs presence on gene was investigated amongst two groups in Iranian population.

Patients have divided into two groups including Alopecia Areata (100), Alopecia totalis and Alopecia Universalis (100) called AA, AT/AU respectively.

4 SNPs were tested for association with Alopecia areata and development of it. Three different analyses were performed: (I) 200 AA/AU/AT cases versus 200 controls (Table 2); (II) 100 AA cases versus 200 controls (Table 3); and (III) 100 AT/AU cases versus 100 AA (Table 4). 4 SNPs were localized in the different regions in the genome (Table 2). They had P-values <0.05 in analyses I (Table 2). However, in analyses II, 4 SNPs showed odd ratio>1, rs1701704 has not demonstrated a significant relationship with AA (Table 3). Of 4 SNPs, 3 SNPs had P-values <0.05 in analyses III, which are localised in *IKZF4*, PRDX5 and *HLA-DQB1*(rs1701704, rs694739 and rs9275572 respectively) (Table 4). The most pivotal SNP of all three analyses was rs9275572 (Chr. 6: 32,678,999bp), which had odd ratio = 5.409 in I analyses.

To confirm the association of the four selected SNPs in the previously pooled discovery, individual genotyping was performed in a sample of 200 AA/AU/AT cases and 200 controls. A step involved 100 AA cases, and 100 AT/AU was done to investigate the influence of the polymorphisms in the development of the disease. Following quality control, 3 SNPs showed P-value with the level of significance. The presence of these 3 SNPs may be a prognosis of the development of the disease to totalis and universalis.

However, in investigation of SNP rs10760706 amongst 100 cases with AA and 100 cases with AT/ AU was not shown any significant association with developing disease (P>0.05; Table 4), frequency of heterozygous of the rs10760706 in the populations were shown a significant association with disease (odd ratio = 3.026, P=0.002).

The present study, Common SNPs of AA was surveyed to avoid the high costs of performing a GWA study. The major histocompatibility complex on chromosome 6p21.3 was identified as a major risk locus for AA. Previous research has implicated various HLA alleles in AA susceptibility. The best-replicated findings have been for alleles of the DRB1 and DQB1 loci (Barahmani et al., 2008; Colombe et al., 1999; Entz et al., 2006). However previous studies were declared there is very unlikely that any genes beyond the HLA region are more significant (Entz et al., 2006; Forstbauer et al., 2012), our research was shown regions on other genes that have a risk for AA. Another strong association was found for the three SNPs (rs1701704, rs10760706 and rs694739) in *IKZF4*, *STX17*, PRDX5 genes.

IKZF4 is a DNA-binding protein binding to the 5GGGAATRCC-3 Ikaros-binding sequence. It May be involved in the development of central and peripheral nervous systems. Essential for the inhibitory function of regulatory T-cells (Treg). Mediates FOXP3-mediated gene silencing in regulatory T-cells (Treg) via recruitment of corepressor CTBP1 (Bloomer et al., 1977).
STX*17* (Syntaxin 17) is a Protein-Coding gene of SNARE of the autophagosome involved in autophagy through the direct control of autophagosome membrane fusion with the lysosome membrane. SNAREs, soluble N-ethylmaleimide-sensitive factor-attachment protein receptors, are essential proteins for fusion of cellular membranes. Diseases associated with *STX17* (rs10760706) include Alopecia Areata (Zhang et al., 2005; Rosengren Pielberg et al., 2008; Petukhova et al., 2010). In the previous study

has indicated *STX17* (rs10760706, P = 3.60 × 10^{-7}, Odds ratio: 1.32) is expressed in the hair follicle and the G allele of rs10760706 is reported to be associated with Alopecia Areata and the grey hair phenotype, which is of interest because AA preferentially attacks pigmented hairs. Risk allele frequency was reported 31.00% (Zhang et al., 2005; Rosengren Pielberg et al., 2008; Petukhova et al., 2010).

rs694739 is a SNP linked to the PRDX5 gene (P = 4.14 × 10^{-7}, Odds ratio: 1.139) (Akar et al., 2002). PRDX5 is an antioxidant enzyme involved in the cellular response to oxidative stress, a process which is dysregulated in AA scalp. It has been implicated in the degeneration of target cells in several autoimmune disorders such as Crohn disease (CD) and Psoriasis (PS) as well as other PRDX family members can serve as autoantigens (Akar et al., 2002, Holley et al., 2007; Wang et al., 2002; Karasawa et al., 2005).

One of the critical points in this study was that the patient group did not suffer from other autoimmune diseases, and individuals with a family history of other autoimmune diseases were excluded from the study to reduce the error in the present review in previous studies. Because of this fact, a number of polymorphisms studied were reported in other autoimmune diseases. As an illustration, rs1701704 was identified loci for type 1 diabetes (Grant et al., 2009; Wang et al., 2010; Lempainen et al., 2013), rs9275572 in HLA-DQB1 gene was reported as Risk alleles for multiple sclerosis (International Multiple Sclerosis Genetics et al., 2007), lupus erythematosus (Hom et al., 2008) and rheumatoid arthritis (Cho et al., 2009). rs694739 in was detected for Crohn disease, psoriasis (Ellinghaus et al., 2012) and MS (Kreft et al., 2017).

In this study, the frequency of allele was no different among females and male's population. In this study, Equal populations were selected in both group (170 females (42.5%) and 230 males (57.5%). However, the frequency of all 4 SNPs in the male's population was higher than the female group in 3 analyses; it was not a significant relationship (P>0.05).

In conclusion, our results suggest that 3 SNPs (rs1701704, rs694739 and rs9275572) in *IKZF4*, PRDX5, *HLA-DQB1* may be a risk factor for the development of AA to other forms (AT/AU) in the Iranian population. The first study is very significant and could be the foundation for further related studies. Some limitations existed in our study. The most significant limitation of this study was lack of access to new case-patients. Although this result might be viewed as supportive evidence, a more detailed workup of these SNPs in large samples is required to allow more definitive conclusions to be drawn.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS CONTRIBUTIONS

Soha Sadeghi conceived of the presented idea and supervised the project,Morteza Taghizadeh and Massoud Houshmand helped supervise the project. Soha Sadeghi, Donya Altafi, Tayebe Talebzade and Iman Alsadat Hosseiniprovided the samples. Golnoosh Taghiabadi, Donya Altafi and Soha Sadeghi reviewed the existing journal's policy. Soha Sadeghi and Donya Altafi performed the statistical analysis. All authors discussed the results and contributed to the writing of the final version of the manuscript. They carried out all experiments.

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