ORIGINAL RESEARCH

Association analysis of the IKZF4 gene with Alopecia Areata in the Chinese Han population

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ABSTRACT

Objective • The IKZF4(Ikaros family zinc finger 4) gene encodes Eos, a zinc finger transcription factor that belongs to the Ikaros family. High expression of Eos on Treg cells is important for the suppression of autoimmune responses and immune homeostasis. It has been suggested that the SNP in IKZF4 may influence the pathogenesis of AA(alopecia areata). The purpose of this study was to explore the relationship between IKZF4 polymorphism and AA in the Chinese Han population.

Methods • We examined 459 patients and 434 controls in this study. The rs1701704 polymorphism was evaluated using HRM analysis and direct sequencing.

Results • The prevalence of the C/C, A/C, and A/A genotypes in AA patients was 7.4%, 37.5% and 55.1%, respectively. There were significant differences in genotype distribution and allele frequencies between AA and the control group ($P < .0001$). The frequency of the C allele in the AA group was significantly higher ($P < .0001$), and the frequencies of the C allele and C/C genotype in patients with family history were higher ($P < .0001$; $P = .001$).

Conclusions • The rs1701704 SNP of IKZF4 may be a genetic marker for assessing the risk of AA in the Chinese Han population. (*Altern Ther Health Med.* 2024;30(10):314-317).

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INTRODUCTION

Alopecia areata is a chronic alopecia disease, that is often characterized by nonscarring, inflammatory, and recurrent. It often appears as localized round or oval patches of hair loss. It also can appear as total hair loss of the scalp (which is called alopecia totalis) or hair loss of the entire body (which is called alopecia universalis).[1] The prevalence of alopecia areata ranges from 0.1% to 0.2% according to different ethnicities and world regions,[2] with a calculated lifetime risk of 1.7%.[3] Alopecia areata can occur at any age, both genders and any ethnic group.[4,5] The prognosis of alopecia areata is variable and cannot be predictable. Many people have conducted research into the pathogenesis of the disease because of the high prevalence of alopecia areata, the enormous psychological impact on patients, and the lack of adequate treatment.[5] There are many hypotheses about the pathogenesis of alopecia areata, which may involve multiple factors ranging from immunology, genetics, and the specificity of autoantigen.[6-12] Currently, hypotheses on the pathogenesis of alopecia areata focus on the impairment of the immune properties of hair follicles and the autoimmune response.[13] Previous studies have reported that Treg cells may be involved in the pathogenesis of alopecia areata.[14]

The IKZF4 (Ikaros family zinc finger 4) gene can encode Eos. Eos is a zinc-finger transcription factor that belongs to the Ikaros family.[15] It was found that Eos can mediate Foxp3-dependent gene signaling in Treg, suggesting that Eos plays an important role in Treg programming.[16] Current study demonstrates that Treg is an important inflammatory cell subset that maintains skin homeostasis and immune privilege.[17] Tregs allow the immune system to differentiate antigens between "self" and "non-self", suppressing the autoimmune reaction and generating the immune reaction against other foreign antigens.[18] Previous study has found the association between the IKZF4 gene (chromosome 12q13) and alopecia areata by Genome-wide association study (GWAS).[19] The rs1701704 variant happened in the IKZF4 domain. This variant is a functional variant, which may cause multiple autoimmune diseases, such as type 1 diabetes [20, 21], and systemic lupus erythematosus.[19] But there were no findings about the relationship between the rs1701704 polymorphism of IKZF4 and alopecia areata in the Chinese

Han population. Studying the relationship between IKZF4 polymorphisms and alopecia areata in the Chinese Han population is significant due to the importance of Treg cells in autoimmune diseases.

MATERIALS AND METHODS

Patients

We conducted a case-control study between January 2017 and January 2019. 459 patients with alopecia areata from the outpatient dermatology clinics of Huashan Hospital Fudan University were included. All alopecia areata patients were Chinese Han population, including 229 males and 230 females, and their mean age was 37.84±13.89 years. All patients were diagnosed with alopecia areata by at least two independent dermatologists. Carefully interrogate and record clinical records of all patients, including age, sex, time of onset, and family history. Inclusion criteria were alopecia areata diagnosed according to the guidelines for alopecia areata.[22] Exclusion criteria included Down syndrome, Turner syndrome, and other autoimmune diseases, such as lupus erythematosus, psoriasis, vitiligo, rheumatoid arthritis, type I diabetes, autoimmune thyroiditis, etc. All patients were divided into several subgroups based on gender(male and female group), severity(severe and mild AA group), age of onset(≤38 years and >38 years group), and family history(with and without family history group). The control group of 434 healthy individuals without any inflammatory or autoimmune disease was recruited to examine whole blood counts, blood glucose levels, kidney and liver function, erythrocyte sedimentation rate, C-reactive protein, anti-streptolysin O titers, rheumatoid factor, Antinuclear antibodies, and venereal disease serum levels were within normal limits. The control group was matched with the alopecia areata group for gender distribution and mean age. This study was approved by the Ethics Committee and was conducted following the Helsinki Declaration of the Code of Ethics for Research. All participants gave written consent to the genetic study and signed an informed consent form.

Extraction of genomic DNA

Genomic DNA was extracted from fresh peripheral blood following the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) instructions.

HRM analysis

We amplified gene fragments surrounding the polymorphism using primer sequences designed to avoid other sequence variations. We designed the primer sequence as follows: forward primer: 5'-GCCCAGCCTTGAGTCATTAT-3', reverse primer: 5'-ACAGCAGCTTTCAGTGTCCA-3'. The PCR cycling was performed in 20 µl volumes containing 200 nM forward primer, 200 nM reverse primer, 200 µM dNTPs, 1x PCR buffer, 5 µM SYTO 9, 0.5 U HotstarTaq DNA polymerase (Qiagen), 2.5 mM MgCl2 and PCR grade water, and 20 ng genomic DNA. The PCR cycling was performed on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif, USA). PCR reaction conditions were: 1 cycle of 95°C for 5 minutes, 45 cycles of 94°C for 10 seconds, 1 cycle of 60°C for 10 seconds, 72°C for 10 seconds, and 72°C for 5 minutes. The HRM was carried out on Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). HRM reaction conditions were: 95°C for 2 minutes, 40°C for 2 minutes, and continuous acquisition from 75°C to 85°C at 1 acquisition per 0.1°C. A standard HRM curve was obtained in each reaction and was used to deduce the genomic type of each AA and healthy sample. To avoid mis-clarification of genotyping between A/A and C/C, exogenous DNA sample with A/A genotype confirmed by sequencing was added (in a 1:1 ratio) to every homozygous samples. Finally, we use the Rotor-Gene 6000 version 1.7 software to analyze the HRM data.

Sequencing

We randomly selected 30 samples containing 10 A/A, 10 C/C, and 10 A/C genotype samples identified by HRM. We use the QIAquick gel purification kit (Qiagen) to perform the gel purification. At last, we use the ABI PRISM 310 gene analyzer (Applied Biosystems, Foster City, CA, USA) to direct sequence the 30 samples.

Statistical analyses

Descriptive statistics were expressed using mean ± standard deviation. Means were compared using the unpaired Student's *t* test. The chi-square test was used to compare genotype and allele frequency distributions. A $P \leq .05$ was considered statistically significant.

RESULTS

Genotyping with HRM and sequencing

We used the Rotor-Gene 6000 system to detect SNPs using amplicon melting analysis in the presence of saturated HRM dyes. HRM analysis can clearly distinguish homozygous (A/A, C/C) and heterozygous (A/C) genomic DNA samples based on the shape of the melting curve. The lower thermal stability of A/A base pairs results in a different melting curve shape compared to A/C base pairs and C/C base pairs. Heterozygous (A/C) genomic DNA samples can be easily distinguished by their biphasic melting pattern (Figure 1). However, sometimes A/A genotypes and C/C genotypes produce indistinguishable curve shapes. To avoid genotype confusion between A/A and C/C, add exogenous DNA samples of the A/A genotype confirmed by direct sequencing to each homozygous sample (at a 1:1 ratio). If the unknown sample is a C/C genotype, the HRM curve shape is the shape of the heteroduplex curve, but if the unknown sample is of the A/A genotype, the shape of the HRM curve will not change. The results of HRM genotyping of all selected samples were completely consistent with those of direct sequencing.

Distribution of IKZF4 genotypes

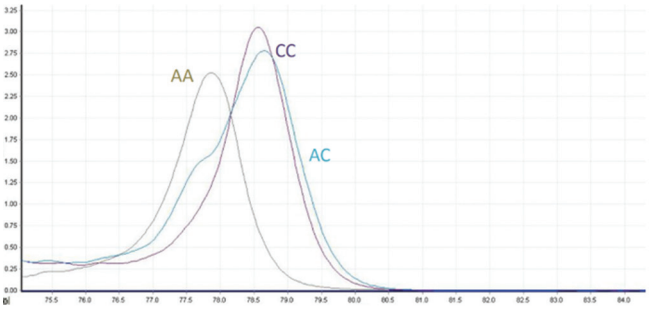
Among the 459 patients, there were 253 cases of A/A genotype with a frequency of 55.1%, 34 cases of C/C genotype with a frequency of 7.4%, and 172 cases of A/C

genotype with a frequency of 37.5%. The frequencies of alleles A and C were 73.9% and 26.1%, respectively. The genotype distribution of rs1701704 SNP was in Hardy-Weinberg equilibrium in both the AA patient group and the healthy control group ($P > .05$). There were statistically significant differences in the distribution of genotypes ($P < .0001$) and alleles ($P < .0001$) between the AA patient group and healthy controls (Table 1).

Table 1. Genotype and allele distribution of the rs1701704 in patients with AA

	Genotype frequencies (%)				Allele frequencies (%)		
Group	C/C	A/C	A/A	*P* value	C	A	*P* value
Male	18(7.9)	81(35.4)	130(56.7)	.641	117(25.5)	341(74.5)	.681
Female	16(6.9)	91(39.6)	123(53.5)		123(26.7)	337(73.3)	
Age ≤38 years	22(9.5)	88(38.1)	121(52.4)	.174	132(28.6)	330(71.4)	.092
Age >38 years	12(5.3)	84(36.8)	132(57.9)		108(23.7)	348(76.3)	
AA	34(7.4)	172(37.5)	253(55.1)	<.0001	240(26.1)	678(73.9)	<.0001
Healthy control	9(2.1)	148(34.1)	277(63.8)		166(19.1)	702(80.9)	
Severe AA	4(4.2)	36(37.5)	56(58.3)	.377	44(22.9)	148(77.1)	.252
Mild AA	30(8.3)	136(37.4)	197(54.3)		196(27.0)	530(73.0)	
With family history	8(12.1)	35(53.0)	23(34.9)	.001	51(38.6)	81(61.4)	<.0001
Without family history	26(6.6)	137(34.9)	230(58.5)		189(24.0)	597(76.0)	

Figure 1. Discrimination of rs1701704 SNP genotype (A/A, A/C, and C/C) using SYTO 9 intercalation dye. Amplification and HRM analysis were performed with the Rotor-Gene 6000 instrument, and genotypes were automatically assigned by the Rotor-Gene software. Dissociation curve analysis of A/A(yellow), A/C(Green) and C/C (purple)type.



IKZF4 genotype and gender, age of onset, severity, or family history

In our study, according to the patient gender, the AA patient group was divided into two subgroups: male group and female group. There were no significant differences in the distribution of genotypes ($P = .641$) and alleles ($P = .681$) (Table 1) between the male and female groups.

We divided patients into two groups based on age of onset: age ≤ 38 years group and age >38 years group. There was a slight difference in genotype ($P = .174$) and allele ($P = .092$) (Table 1) distribution between the two groups, but the difference did not reach statistical significance.

Severity was assessed based on the most severe condition the patient has presented since onset. Patients were divided into four subgroups according to the AA study evaluation guidelines: patients with patchy AA, patients with alopecia totalis (AT), patients with alopecia totalis/alopecia universalis (AT/AU), and patients with alopecia universalis (AU).[22] In this study, mild AA refers to patients with patchy AA (n = 363), while severe AA refers to patients with AT, AT/AU, and AU (n = 96). Based on the above, these patients were divided into two groups: the severe AA group and the mild AA group. The difference in the distribution of genotypes ($P = .377$) and alleles ($P = .252$) between the severe AA group and the mild AA group was not statistically significant (Table 1).

In our study, patients were considered to have a family history of alopecia areata if more than one first- or second-degree relative in the family had alopecia areata. Based on family history, these patients were divided into two groups: those with an AA family history and those without an AA family history. 66 cases had a family history of AA. There were significant differences in the distribution of genotypes ($P = .001$) and alleles ($P < .0001$) between the two groups (Table 1). The frequency of the C allele in the AA group with family history was higher.

DISCUSSION

Genetic factors play an important role in the pathogenesis of AA. Research has found that genetic factors predispose some humans to some autoimmune diseases.[11,19,23] There is a growing interest in the study of genes that may confer risk.

Eos, encoded by the IKZF4 gene, is a zinc finger transcription factor belonging to the Ikaros family. Eos is highly expressed in regulatory T cells with transcriptional repressive activity.[11,19,23-25] Studies have shown that Eos can interact with C-terminal binding protein 1 (CtBP1).[24] Eos can directly interact with Foxp3 and is involved in gene silencing.[16,26] CtBP1 has been shown to recruit corepressor complexes to modify chromatin structure, thereby silencing gene expression.[27-29] Eos and CtBP1 are required for histone modification and promoter methylation involved in selective gene silencing in regulatory T cells. Knockout of Eos abolishes the ability of Treg cells to suppress immune responses in vitro and in vivo and is endowed with partial effector functions.[16,26] Alopecia areata is currently considered to be an autoimmune disease mediated by T cells, and the above conclusions are consistent with this view.

Recently, Petukhova et al. reported IKZF4 as a novel susceptibility locus for alopecia areata in a North American population genome-wide association study (GWAS).[19] However, there was no other report about the relationship between the rs1701704 polymorphism of IKZF4 and the Chinese AA patients. Our study was the first time to explore the association.

In our case-control design, we investigated the association between a single nucleotide polymorphism (rs1701704) of IKZF4 and AA in the Chinese Han population. The report on the correlation between IKZF4 polymorphism and AA is the first time in China. Our findings provide strong evidence that IKZF4 is involved in the pathogenesis of AA, especially in patients with a family history.

Our study found a significant difference in the genotype distribution between AA patients and healthy control individuals ($P < .0001$). The frequencies of A and C alleles were significantly different between AA patients and healthy control

individuals ($P < .0001$). The C/C genotype and C allele frequencies in the AA patient group were higher than those in the healthy control group. Furthermore, we found that this locus was more strongly associated with patients with a family history of alopecia areata ($P = .001$). The C allele and C/C genotype are more common in patients with a family history of alopecia areata. The results indicated the association of the rs1701704 polymorphism of IKZF4 with family history of alopecia areata. Therefore, this SNP may be used to predict the risk of acquiring alopecia areata in Chinese populations, especially for those with a family history. For the variation of the rs1701704 gene, our results found no correlation between different gender groups ($P = .641$) and age of onset groups ($P = .174$). This finding suggests that the rs1701704 SNP of IKZF4 plays an important role in the etiology of AA, independent of gender and age. Our study found no significant difference in the genotype distribution between the severe AA group and the mild AA group ($P = .377$). That suggests that the rs1701704 SNP of the IKZF4 gene may not be related to the severity of alopecia areata.

In order to correctly distinguish between homozygous C/C type and homozygous A/A type, the determined A/A type DNA sample was added to each homozygous sample as an internal standard,[30, 31] if there were no additional peaks on the dissociation curve, it indicated The genotype of this sample is A/A.

In conclusion, the study found that the rs1701704 single nucleotide polymorphism was associated with the etiology and pathogenesis of AA in the Chinese Han population.

ETHICAL COMPLIANCE

This study was approved by the ethics committee of Hushan Hospital, Shanghai Medical College, Fudan University. Signed written informed consent was obtained from the patients and/or guardians.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report relevant to this article.

AUTHOR CONTRIBUTIONS

YM and QY designed the study and performed the experiments, SQ and RH collected the data, SQ, RH and YS analyzed the data, and YM and QY prepared the manuscript. All authors read and approved the final manuscript.

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