Association analysis of IKZF4 gene with alopecia areata in Chinese Han population

Ying Miao
Department of Dermatology, Hushan Hospital, Shanghai Medical College, Fudan university
Ruiming Hu
Department of Dermatology, Hushan Hospital, Shanghai Medical College, Fudan university
Youyu Sheng
Department of Dermatology, Hushan Hospital, Shanghai Medical College, Fudan university
Sisi Qi
Department of Dermatology, Hushan Hospital, Shanghai Medical College, Fudan university
Qinping Yang (qpyang_shhs1@126.com)
Department of Dermatology, Hushan Hospital, Shanghai Medical College, Fudan university

Research Article

Keywords: alopecia areata, IKZF4, rs1701704, Single nucleotide polymorphism

Posted Date: March 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1423386/v2

Abstract

Background: The IKZF4 gene encodes Eos, a zinc finger transcription factor that belongs to the Ikarosfamily. 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.

Aims and Objectives: The purpose of this study was to explore the relationship between IKZF4 polymorphism and AA in Chinese Han population.

Materials and Methods: In this study, we examined 459 patients and 434 controls using HRM analysis and direct sequencing to assess.the rs1701704 polymorphism..

Results: The prevalence of the C/C, A/C and A/A genotypes was 7.4%, 37.5% and 55.1%, respectively. There were significant differences in genotype distribution and allele frequencies between AA and control group ($p<0.0001$). The frequency of C allele in AA group was significantly higher($p<0.0001$), and the frequencies of C allele and C/C genotype in patients with family history were higher ($p<0.0001$;$p=0.001$;).

Conclusions: The rs1701704 SNP of IKZF4 may be a genetic marker for assessing the risk of AA in Chinese Han population.

Introduction

Alopecia areata (AA) is a nonscarring inflammatory chronic recurrent alopecia disease characterized by localized circular or oval hair loss, which can progress to severe forms with total hair loss of the scalp (alopecia totalis) or the entire body (alopecia universalis)[1]. Depending on ethnic background and area of the world, the prevalence of alopecia areata is 0.1 to 0.2%[2], with a calculated lifetime risk of 1.7%[3]. AA affects both sexes equally, patients of all ages, and all ethnic groups[1, 4, 5]. The prognosis of AA is unpredictable and highly variable. The relatively high prevalence of the disease, its overwhelming psychological impacts, and the lack of sufficient treatment prompt a thorough investigation of the disease pathogenesis[6]. There are many hypotheses for the etiopathogenesis of AA and it is probable that it involves several factors ranging from the genetics of the immune system to autoantigen specificity and expression patterns[7–13]. Presently, the hypotheses for AA development mostly focus on the damage of immune properties of the hair follicles and the nature of autoimmune reactions that results in the lymphocytes' subsequent attack[7]. Some previous study reported the possilbe involvement of Treg cells in the pathogenesis of AA[14–16].

The IKZF4 gene encodes Eos, a zinc-finger transcription factor of the Ikaros family. Eos has been suggested to mediate Foxp3-depengdent gene signaling in Tregs, implicating Eos' critical role in Treg programming[17]. Treg has emerged as a key inflammatory cell subsets in maintaining cutaneous homeostasis[18] and immune privilege[19]. Treg allows the immune response to distinguish between "self"

and “non-self” antigens, inhibiting autoimmune response (peripheral self-tolerance) and managing immune response against exogenous antigens[20]. The association between the IKZF4 gene (chromosome 12q13) and AA was detected by GWA scanning[21]. The rs1701704 variant in the domain of IKZF4 is a functional variant that can lead to a variety of autoimmune diseases, including type 1 diabetes[22, 23] and systemic lupus erythematosus[21]. According to the important roles of Treg cell in autoimmune diseases, it would be meaningful to study the relationship between IKZF4 polymorphism and alopecia areata in the genetic homogeneous population of Han Chinese, in order to clarify whether the gene plays a key role in autoimmune diseases.

Materials And Methods

Patients

A case-control study carried on between January 2017 and January 2019, including 459 participants from the Outpatient Dermatology Clinic. Alopecia areata patients were Chinese population (229 males and 230 females, with an average age of 37.84 ± 13.89 years). At least two independent dermatologists diagonse all the patients as alopecia areata. Detailed clinical records of all patients, including age, gender, time of onset and family history, have been obtained. The inclusion criterion were diagnosis of alopecia areata according to the alopecia areata guidline[24]. Exclusion criteria included Down syndrome and Turner syndrome. According to gender, severity, age of onset, and family history, all patients were classified into several subgroups. The control group consisted of 434 healthy individuals without any inflammatory or autoimmune diseases, and the whole blood count, blood glucose level, renal and liver function, erythrocyte sedimentation rate, C-reactive protein, antistreptolysin O titre, rheumatoid factor, antinuclear antibodies and STD serum levels were within the normal range. The gender distribution and average age of the controls were matched with the patients. This study was approved by the Ethics Committee and carried out in accordance with the declaration of Helsinki research ethics code. All participants agreed in writing to conduct genetic research.

Extraction Of Genomic Dna

Accoring to the instructions of the kit, genomic DNA was extracted from the fresh peripheral blood using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA).

Hrm Analysis

The PCR cycling was carried on GeneAmp PCR System 9700 (Applied Biosystems), and the HRM was carried on Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). The designed primer sequences could be used to amplify the gene fragment around the polymorphism and avoid other sequence variats The primer sequences were as follows: the forward primer: 5-

GCCCAGCCTTGAGTCATTAT-3′, the reverse primers: 5′-ACAGCAGCTTTCAGTGTCCA-3′. The PCR cycling was performed in a 20 μl volume, which contained 20 ng of genomic DNA, 200 nM of each primer, 200 μM of dNTPs, 0.5 U HotstarTaq DNA polymerase, 1x polymerase chain reaction (PCR) buffer, 5 μM SYTO 9, 2.5 mM $MgCl_2$, and PCR-grade water by use of the HotstarTaq DNA polymerase (Qiagen). Each reaction was repeated three times. The reaction conditions of PCR were set as follows: 1 cycle of 95 ºC for 5 minutes, 45 cycles of 94 ºC for 10 seconds, 60 ºC for 10 seconds, and 72 ºC for 10 seconds, 1 cycle of 72 ºC for 5 minutes; followed by an HRM step of 95 ºC for 2 minutes, 40 ºC for 2 minutes, and continuous acquisition from 75 ºC to 85 ºC at 1 acquisition per 0.1 ºC. A standard HRM curve was obtained in each reaction and was used to deduce the genomic type of each AA and normal sample. The HRM data were analyzed by the Rotor-Gene 6000 1.7 software.

Sequencing

We selected thirty samples randomly which contained 10 A/A, 10 C/C and 10 A/C genotype samples identified by HRM. The thirty samples were directly sequenced. Gel purification of amplicons was carried out by QIAquick gel purification kit (Qiagen). The amplified products were sequenced by the ABI PRISM 310 gene analyzer (Applied Biosystems, Foster City, CA).

Statistical Analyses

The mean value ± standard deviation is used to express descriptive statistics. Unpaired Student's t test was used for comparison of means. Chi-squared test was used to compare genotype and allele frequency distribution. It was considered statistically significant that P values was less than or equal to 0.05.

Results

Genotyping with HRM and sequencing

We used amplicon melting analysis in the presence of the saturated HRM dye by the Rotor-Gene 6000 System to detect SNP. HRM analysis can clearly distinguish between the homozygous (A/A, C/C) and heterozygous (A/C) genomic DNA samples according to the different melting curve shapes. The different melting curve shapes were caused by the lower thermal stability of A/A base pairs compared with A/C base pairs and C/C base pairs. Heterozygous (A/C) genomic DNA samples can be easily distinguished by their unique biphasic melting patterns(Fig 1). However, sometimes the A/A genotype and C/C genotype may produce the similar curve shape. To avoid misclarification of genotypes between A/A and C/C, the foreign DNA sample of A/A genotype confirmed by directly sequencing was added to every homozygous samples (in a 1:1 ratio). If the unknown samples are A/A genotype, the melting curve shapes will not be changed after adding the foreign A/A genotype DNA sample. If the unknown samples are C/C genotype, the melting curve shapes were the heteroduplexes curve shapes, and then, the samples can be correctly

identified. The genotyping results of all selected samples by HRM was 100% identical with those obtained by direct sequencing.

Distribution of IKZF4 genotypes

Among the 459 patients, the A/A genotype had 253 cases ,the frequency was 55.1%, the C/C genotype had 34 cases ,the frequency was 7.4%, and the A/Cgenotype had 172 cases, the frequency was 37.5%. The frequency of allele A and C was 73.9% and 26.1% respectively. In both AA patient group and healthy control group, the genotype distributions of SNP rs1701704 were in Hardy-Weinberg equilibrium. The distribution of polymorphism was assessed in the patient and control groups. The frequencies of SNP were in Hardy-Weinberg equilibrium ($p>0.05$). Differences in the distribution of both genotype ($p<0.0001$) and alleles ($p<0.0001$) between AA patient group and healthy control group were statistically significant (Table 1).

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

Group	Genotype frequencies (%)			p	Allele frequencies(%)		p
	C/C	A/C	A/A		C	A	
Male	18(7.9)	81(35.4)	130(56.7)	0.641	117(25.5)	341(74.5)	0.681
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)	0.174	132(28.6)	330(71.4)	0.092
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)	0.0001	240(26.1)	678(73.9)	0.0001
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)	0.377	44(22.9)	148(77.1)	0.252
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)	0.001	51(38.6)	81(61.4)	0.0001
Without family history	26(6.6)	137(34.9)	230(58.5)		189(24.0)	597(76.0)	

IKZF4 genotype and severity or family history

In our study, we divided the patients into two groups according to their gender: a male group and a female group. There was no significant difference in the distribution of genotype ($p=0.641$) and alleles ($p=0.681$) (Table 1) between the two groups.

We divided the patients into two groups according to the onset ages,: Age ≤38 years group and Age >38 years group. There was notable differences between the two groups in the distribution of genotype (p=0.174) and alleles (p=0.092). But there was a little different between the two groups.

The assessment of severity was based on the patient's most severe condition since the onset of alopecia areata. According to the AA investigational assessment guidelines, patients were classified into four subgroups: patients having patchy AA,patients having alopecia totalis (AT), patients having alopecia totalis/universalis (AT/AU), and patients having alopecia universalis (AU)[24]. In this study, patients with patchy AA were classified as mild AA (n=363), while patients with AT, AT/AU, AU were classified as severe AA (n=96). According to the severity of alopecia areata, those patients were divided into two groups: a severe AA group (AT, AU and AT/AU) and a mild AA group (patchy AA). There was no significant difference in the distribution of both genotype (p=0.377) and alleles (p=0.252) between severe AA group and mild AA group (Table 1).

In our study, the family history was defined as at least one firee-degree or second-degree relative having alopecia areata. There were 66 patients having the family history of AA. According to the family history, those patients were also divided into two different groups: patients with family history group and patients without family history group. Differences in the distribution of both genotype(p=0.001) and alleles (p<0.0001) between these two groups reached the statistical siginificance (Table 1) . The frequency of C allele in the group with family history was higher than that in the group without family history.

Discussion

Genetic factors play an important role in pathogenesis of AA. There is a number of evidence that genetic risk factors predispose humans to this autoimmune disorder[12, 21, 25]. There is an growing interest in studying genes that can bring moderate risk.

The IKZF4 gene encodes Eos, a zinc finger transcription factor that belongs to the Ikarosfamily. Eos is highly expressed in Treg cells and is known to have transcrption repression activity[12, 21, 25–27]. Eos interacts directly with Foxp3 and is necessary for gene silencing without affecting expression of Foxp3 activated genes[17, 28]. Eos is known to interact with C-terminal binding protein 1 (CtBP1)[26], which has been shown to recruit corepressor complexes to modify chromatin structures and silence gene expression[29–31]. Eos and its corepressor CtBP1 are necessary for histone modifications and ultimately promoter methylation involved in selective gene silencing in Treg. Knockdown of Eos in Treg abrogates their ability to suppress immune responses in vitro and in vivo and endows them with partial effector function[17, 28]. The above conclusion are consistent with the hypothesis that Alopecia Areata is an autoimmune disease mediated by T cells.

Recently, IKZF4 was repored by Petukhova et al. as a new susceptibility locus in a genome-wide association study(GWAS) of North American population[21]. In our case-control design, we studied the relationship between the single nucleotide ploymorphism(rs1701704) of IKZF4 and AA in Chinese

population. It is the first report on the association between IKZF4 polymorphism and AA in China. The result of our study provide strong evidence that IKZF4 is involved in the pathogenesis of AA, especially among the patients with family history.

There was a significant difference in the distribution of genotype between AA patients and healthy control individuals (p = 0.000). There were significant difference in the frequencies of A and C allele between AA patients and healthy control individuals (p = 0.000). The frequencies of C/C genotype and C allele and were higher in the AA patients group than in the healthy control individuals group. In addition, we were able to prove that the correlation was stronger in those patients with family history (p = 0.001). C alleles and C/C genotypes were more frequent in patients with family history than in patients without family history. This reveals the correlation between rs1701704 polymorphism of IKZF4 and the family history of alopecia areata. Therefore, this SNP marker may contribute to the stratification of AA risk in Chinese population, particularly for those with family history. For the variant of rs1701704 gen, our results found no correlation between the subgroups of different gender groups (p = 0.641) and age groups (p = 0.174). This finding suggests that rs1701704 single necletiode polymorphism of IKZF4 plays an important role in aetiology of AA, regardless of gender and age.

However, when the base pair is inverted or neutral (A:T to T:A or G:C to C:G), not all homozygotes can be distinguished by curve shape or Tm. In this case, a known homozygote can be mixed into each unknown homozygote, and then the mixture can be melted again for complete genotyping.

To rule out the possibility that homozygous C/C type and homozygous A/A type could not be accurately distinguished, the DNA sample of A/A type was added to each homozygous sample as internal standard[32, 33], there was no additional peak on the dissociation curve, indicating that the sample belonged to the A/A type.

In conclusion, the study found that the rs1701704 sindle nucleotide polymorphism was related to the etiology and pathogenesis of AA in Chinese population.

Abbreviations

AA Alopecia Areata

Treg $CD4^+CD25^+$ regulatory T cells

HRM High-resolution melting

GWAS Genome-wide association study

PCR Polymerase chain reaction

SNP Single nucleotide polymorphism

Declarations

Ethics approval and consent to participate: This study was approved by the Ethics Committee of Huashan Hospital Fudan University and conducted in accordance with the declaration of Helsinki guidelines for ethics in research. All participants gave written informed consent for genetic studies.

Consent for publication: All authors have read the article and agree to publish.

Availability of data and material: All material and data are true and reliable.

Competing interests: No

Funding: No Funding

Authors' contributions: Y. M, Y.Y.S and Q.P.Y studied. Y.M and Q.P.Y designed the study. Y.M, Y.Y.S, S.S.Q and R.M.H provide the necessary reagents. Y.M, Y.Y.S, S.S.Q, R.M.H and Q.P.Y diagnosed alopecia areata., Y.M and Y.Y.S analyzed the data. Ying Miao wrote the paper.

All authors have read and approved the final manuscript.

Acknowledgements: This work was supported in part by the Central Laboratory of Shanghai Huashan Hospital

Conflicts of interest: None.

References

1. journals.lww.com.
2. Safavi K. Prevalence of alopecia areata in the First National Health and Nutrition Examination Survey[J]. Arch Dermatol, 1992,128(5):702.
3. Betz R C, Petukhova L, Ripke S, et al. Genome-wide meta-analysis in alopecia areata resolves HLA associations and reveals two new susceptibility loci[J]. Nat Commun, 2015,6:5966.
4. Alzolibani A A. Epidemiologic and genetic characteristics of alopecia areata (part 1)[J]. Acta Dermatovenerol Alp Pannonica Adriat, 2011,20(4):191-198.
5. Strazzulla L C, Wang E, Avila L, et al. Alopecia areata: Disease characteristics, clinical evaluation, and new perspectives on pathogenesis[J]. J Am Acad Dermatol, 2018,78(1):1-12.
6. Rajabi F, Drake L A, Senna M M, et al. Alopecia areata: a review of disease pathogenesis[J]. Br J Dermatol, 2018,179(5):1033-1048.
7. Wang E, McElwee K J. Etiopathogenesis of alopecia areata: Why do our patients get it?[J]. Dermatol Ther, 2011,24(3):337-347.
8. Brzezinska-Wcislo L, Bergler-Czop B, Wcislo-Dziadecka D, et al. New aspects of the treatment of alopecia areata[J]. Postepy Dermatol Alergol, 2014,31(4):262-265.

9. Islam N, Leung P S, Huntley A C, et al. The autoimmune basis of alopecia areata: a comprehensive review[J]. Autoimmun Rev, 2015,14(2):81-89.
10. Perera E, Yip L, Sinclair R. Alopecia areata[J]. Curr Probl Dermatol, 2015,47:67-75.
11. Dainichi T, Kabashima K. Alopecia areata: What's new in epidemiology, pathogenesis, diagnosis, and therapeutic options?[J]. J Dermatol Sci, 2017,86(1):3-12.
12. Petukhova L, Christiano A M. The genetic architecture of alopecia areata[J]. J Investig Dermatol Symp Proc, 2013,16(1):S16-S22.
13. Biran R, Zlotogorski A, Ramot Y. The genetics of alopecia areata: new approaches, new findings, new treatments[J]. J Dermatol Sci, 2015,78(1):11-20.
14. Han Y M, Sheng Y Y, Xu F, et al. Imbalance of T-helper 17 and regulatory T cells in patients with alopecia areata[J]. J Dermatol, 2015,42(10):981-988.
15. Hamed F N, Astrand A, Bertolini M, et al. Correction: Alopecia areata patients show deficiency of FOXP3+CD39+ T regulatory cells and clonotypic restriction of Treg TCRbeta-chain, which highlights the immunopathological aspect of the disease[J]. PLoS One, 2019,14(9):e222473.
16. Speiser J J, Mondo D, Mehta V, et al. Regulatory T-cells in alopecia areata[J]. J Cutan Pathol, 2019,46(9):653-658.
17. Pan F, Yu H, Dang E V, et al. Eos mediates Foxp3-dependent gene silencing in CD4+ regulatory T cells[J]. Science, 2009,325(5944):1142-1146.
18. Dudda J C, Perdue N, Bachtanian E, et al. Foxp3+ regulatory T cells maintain immune homeostasis in the skin[J]. J Exp Med, 2008,205(7):1559-1565.
19. Matzinger P. The danger model: a renewed sense of self[J]. Science, 2002,296(5566):301-305.
20. Sakaguchi S, Yamaguchi T, Nomura T, et al. Regulatory T cells and immune tolerance[J]. Cell, 2008,133(5):775-787.
21. Petukhova L, Duvic M, Hordinsky M, et al. Genome-wide association study in alopecia areata implicates both innate and adaptive immunity[J]. Nature, 2010,466(7302):113-117.
22. Koskinen M K, Mikk M L, Laine A P, et al. Longitudinal Pattern of First-Phase Insulin Response Is Associated with Genetic Variants Outside the Class II HLA Region in Children with Multiple Autoantibodies[J]. Diabetes, 2019.
23. Lempainen J, Harkonen T, Laine A, et al. Associations of polymorphisms in non-HLA loci with autoantibodies at the diagnosis of type 1 diabetes: INS and IKZF4 associate with insulin autoantibodies[J]. Pediatr Diabetes, 2013,14(7):490-496.
24. Olsen E A, Hordinsky M K, Price V H, et al. Alopecia areata investigational assessment guidelines–Part II. National Alopecia Areata Foundation[J]. J Am Acad Dermatol, 2004,51(3):440-447.
25. Martinez-Mir A, Zlotogorski A, Gordon D, et al. Genomewide scan for linkage reveals evidence of several susceptibility loci for alopecia areata[J]. Am J Hum Genet, 2007,80(2):316-328.
26. Perdomo J, Crossley M. The Ikaros family protein Eos associates with C-terminal-binding protein corepressors[J]. Eur J Biochem, 2002,269(23):5885-5892.

27. Li S, Yao W, Pan Q, et al. Association analysis revealed one susceptibility locus for vitiligo with immune-related diseases in the Chinese Han population[J]. Immunogenetics, 2015,67(7):347-354.
28. gxreferences.blogspot.com.
29. Shi Y, Sawada J, Sui G, et al. Coordinated histone modifications mediated by a CtBP co-repressor complex[J]. Nature, 2003,422(6933):735-738.
30. Koipally J, Georgopoulos K. A molecular dissection of the repression circuitry of Ikaros[J]. J Biol Chem, 2002,277(31):27697-27705.
31. Hu R, Sharma S M, Bronisz A, et al. Eos, MITF, and PU.1 recruit corepressors to osteoclast-specific genes in committed myeloid progenitors[J]. Mol Cell Biol, 2007,27(11):4018-4027.
32. Garritano S, Gemignani F, Voegele C, et al. Determining the effectiveness of High Resolution Melting analysis for SNP genotyping and mutation scanning at the TP53 locus[J]. BMC Genet, 2009,10:5.
33. Liew M, Pryor R, Palais R, et al. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons[J]. Clin Chem, 2004,50(7):1156-1164.

Figures

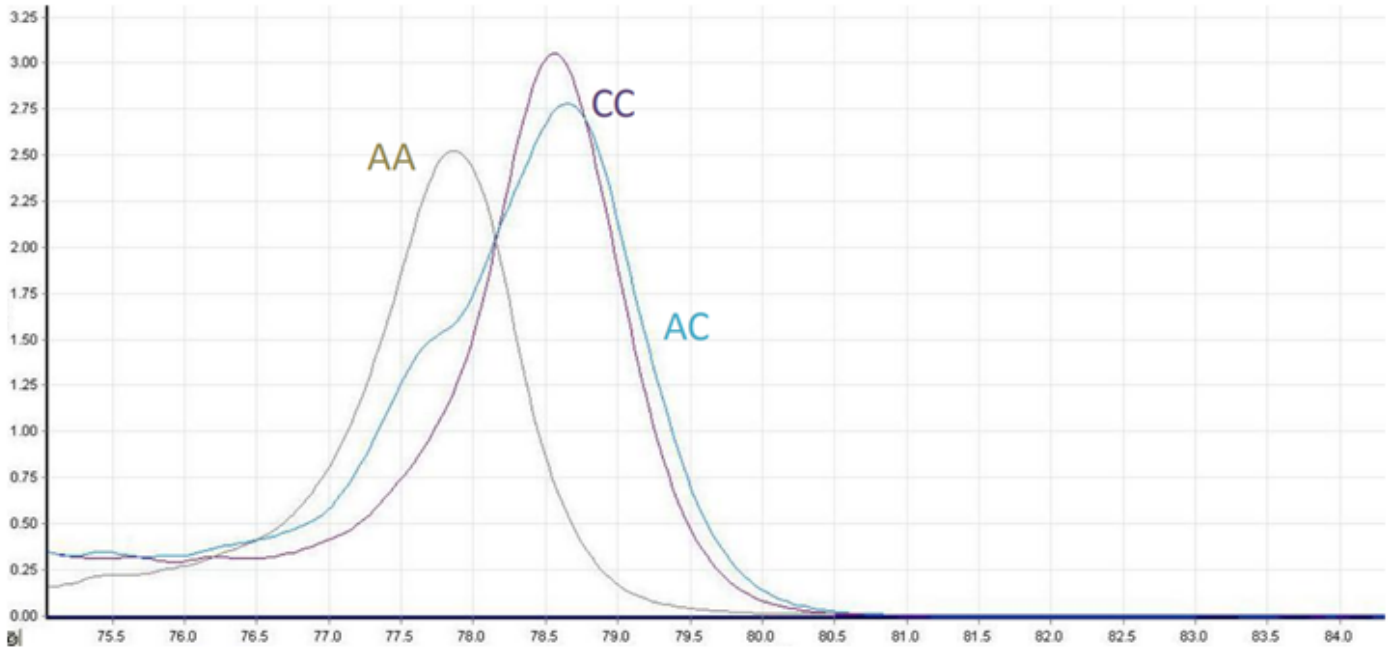

Figure 1

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.