Exomic Sequencing of Immune-Related Genes Reveals Novel Candidate Variants Associated with Alopecia Universalis

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Abstract

Alopecia areata (AA) is a common autoimmune disorder mostly presented as round patches of hair loss and subclassified into alopecia totalis/alopecia universalis (AT/AU) based on the area of alopecia. Although AA is relatively common, only 5% of AA patients progress to AT/AU, which affect the whole scalp and whole body respectively. To determine genetic determinants of this orphan disease, we undertook whole-exome sequencing of 6 samples from AA patients, and 26 variants in immune-related genes were selected as candidates. When an additional 14 AU samples were genotyped for these candidates, 6 of them remained at the level of significance in comparison with 155 Asian controls (p<1.92×10⁻⁵). Linkage disequilibrium was observed between some of the most significant SNPs, including rs41559420 of HLA-DRB5 (p<0.001, OR 44.57) and rs28362679 of BTNL2 (p<0.001, OR 30.21). While BTNL2 was reported as a general susceptibility gene of AA previously, HLA-DRB5 has not been implicated in AA. In addition, we found several genetic variants in novel genes (HLA-DQB, TLR1, and PMS2) and discovered an additional locus on HLA-A, a known susceptibility gene of AA. This study provides further evidence for the association of previously reported genes with AA and novel findings such as HLA-DRB5, which might represent a hidden culprit gene for AU.

Introduction

Alopecia areata (AA) is characterized by autoimmune-mediated patchy hair loss. The alopeic patches can be multiple and coalesce to diffuse hair loss over the scalp, even the whole body. Severe forms of AA are named based on the body area affected by alopecia and include alopecia totalis (AT; affecting the whole scalp) and alopecia universalis (AU; affecting the entire body). Although AA is fairly common, with the life-time risk being 1.7% [1], progression to AT/AU is rare and only reported in 5% of AA patients [2]. Clinically, the extent of hair loss is the most important prognostic factor in AA [3]. Although more than 50% of classic AA patients recover without treatment [4], less than 10% of patients with AT/AU recover fully [5].

The underlying etiopathogenesis of AA has been revealed as an autoimmune mechanism from multiple lines of studies. Histologically, AA is characterized by immune infiltrates surrounding the hair follicle. Moreover, AA is responsive to immunosuppressive therapies, further supporting an autoimmune basis for the disease [6]. In addition, several genetic studies on AA have shown that immune factors are key genetic components of AA. Early association studies with selected candidate genes revealed that AA or AT/AU is associated with several immune-related genes including HLA-DQB1, MICA, PTPN22, IL1RN, AIRE, and TRAF1/C5 [7–14]. Furthermore, HLA-DQB1, MICA, PTPN22, IL1RN, and TRAF1/C5 were found to influence disease severity [7,9–11,13,14]. Genetic susceptibility loci harboring murine homologues of HLA have also been discovered in a C3H/HeJ mouse model of AA [15]. In addition, recent genome-wide linkage and association studies have identified several susceptibility loci near, or within, genes related to autoimmunity, such as MICA and BTNL2 [16–18].

Although previous studies have identified AA as an immune-related disorder and have shown several genetic loci throughout the genome, association studies for the most severe type of AA (AU) have rarely been performed, especially using next-generation
sequencing technology. Most rare missense genetic variations are predicted to be deleterious and, thus, association studies with these variants may be helpful in this regard [19]. Recent studies have also suggested that the majority of variants seem to be rare, and these rare variants are likely to be key determinants for the health status of individuals [20,21]. Given that AU patients are rare and have the most severe AA phenotype, identification of rare variants with large effect sizes would be a good strategy to further elucidate the genetic basis for this disease.

In this study, on the basis of the immune-related mechanism of the disease, we conducted an exome-sequence analysis of immune-related genes in AU patients with the aim of identifying genetic determinants of AU by focusing on rare and pathogenic candidates.

Materials and Methods

Ethics statement

Ethical approval for the study was obtained from the relevant Ethics Committees (The Institutional Review Board of the Seoul National University Hospital). This study was conducted according to Declaration of Helsinki principles. Written informed consent was obtained before blood was taken from patients with AU.

Study sample selection and DNA extraction

For this study, 20 patients with AU were recruited from the outpatient clinic of Seoul National University Hospital. Overall demographic data and clinical features are summarized in Table 1, and detailed individual data are presented in Table S1. A diagnosis of AU was based on any experience of whole body hair loss in the participant’s lifetime, and involved areas other than the scalp were eyebrow, axillary hair, and pubic hair (eyebrow only in child). Genomic DNA was extracted from peripheral blood by using QuickGene DNA whole blood kit with QuickGene-610L equipment (Fujifilm, Tokyo, Japan). DNA concentrations were measured by Nanodrop ND-1000 spectrophotometer (Nano-drop Technologies, Delaware, USA).

Table 1. Demographic data of enrolled patients.

| Age, years | 3–43 (mean: 15) |
| Gender, n | Male 13 (65%) |
|           | Female 7 (35%) |
| Onset, n\(^1\) | Early onset 15 (75%) |
|           | Late onset 5 (25%) |
| Disease duration, years | 1–18 (mean: 5.5) |
| Nail dystrophy, n | 6 (30%) |
| Family history, n | 6 (30%) |
| Comorbid disorders, n | Atopy 2 (10%) |
|           | Thyroid disease 2 (10%) |
|           | Psoriasis 1 (5%) |

\(^1\) Based on the age at onset of < or \(\geqslant\)13 yr, patients were classified to the early-onset group.

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Whole-exome sequencing of 6 individuals with alopecia universalis

Exomic regions of DNA were captured by SureSelect Human All Exon v.2 Kits (Agilent Technologies, California, USA) and sequenced through HiSeq 2000 (Illumina, California, USA) in 6 samples among study subjects. All procedures followed the manufacturer’s instructions. The generated reads were aligned to the NCBI human reference genome build 37 (hg19) using the Genomic Short-read Nucleotide Alignment Program alignment tool [22]. Only positions having \(\geq 8\) uniquely aligned reads and \(\geq 20\) mean quality score (Q score) were carried forward for further analyses. Variant calling was performed according to the criteria of our previous study [23], and all the variants called were annotated by the RelSeq gene set and compared with dbsNP build 132.

Filtration into functional candidate variants

In this study, we focused on the variants found in immune-related genes. Candidate genes were categorized by gene ontology by using DAVID [24] and only variants in immune-related genes were collected. Among them, we selected nonsynonymous single nucleotide polymorphisms (SNPs), coding sequence insertions/deletions (indels), and canonical splice-site variants as pathogenic candidates.

For further filtration, genotype frequencies of pathogenic candidates were compared between patient and control groups in both dominant and recessive models. The control samples used for this study were as follows: (1) 59 Korean samples (3 from whole-genome sequencing (WGS), 41 from whole-exome sequencing (WES)), (2) 56 Asian HapMap samples (10 from WGS, 46 from WES), and (3) 40 Mongolian samples from WES. These controls were selected from the Asian Genome Project of our group, and 58 of them were published in our previous studies [25,26]. Statistical comparisons were drawn using a non-parametric method of Fisher’s exact test, and a p-value less than 0.05 was used as a standard for the candidate selection. To reduce the number of variants selected by chance, we further narrowed our candidates into those shown in at least 2 patient samples.

Finally, we manually checked the coverage depth of each variant and excluded variants showing deviated patterns of coverage. The deviated pattern of coverage was defined as a coverage depth ratio between the reference and variant alleles that is either \(>0.5\) or \(<0.5\) throughout the samples (Table S2). Overall filtration steps above were summarized in Figure S1. In addition to the candidates selected, rs28362679 in \(BTNL2\) was included since it is not only associated with MHC class II but also previously highlighted as a susceptibility gene for the development of AA [17].

Further genotyping in 14 additional alopecia universalis patients and frequency comparison with control samples

The remaining variants were genotyped in the other 14 patient samples with PCR and subsequent Sanger sequencing (Table S3). Using Fisher’s exact test, the genotype frequency of each variant was compared with that of 155 control samples in both dominant and recessive models. For both analyses, the threshold level of significance was set at a p-value lower than 1.92 \(\times 10^{-3}\), considering multiple testing of our selected candidates (n = 26). For reference, the allele frequency of each variant in Asians was also checked against the 1000 Genomes Project by using the ENGINES program [27].
Linkage disequilibrium analysis

We explored the linkage disequilibrium (LD) structures among final candidates to check if there were any crucial haplotypes associated with AU. The genotypes of all 20 patient samples were used for LD estimation, which was calculated by the Haploview program (Version 4.2). We defined LD structure between SNPs when an $r^2$ value was higher than 0.3, and pairwise comparisons of markers $>500$ kb apart were ignored.

Results

We sequenced exomic regions of 6 individuals with AU and aligned the generated reads to the NCBI human reference genome build 37 (Table 2). The percentage of covered bases in the bait region was 84.7% on average (76.2–88.75%) with the mean coverage depth of 69.1 × (43.6–100.0 ×). SNPs and short indels were called according to the criteria set out in our previous study, and annotated based on the ReSeq gene set and dbSNP build 132 [23]. The annotated SNPs and indels were filtered stepwise as described in the methods, and narrowed down to 25 SNPs and 1 indel by excluding variants showing ambiguous patterns of coverage depth in exome sequencing and a variant with PCR failure (Table 3).

Next, we genotyped the final 26 candidates in additional 14 AU samples by using PCR and subsequent Sanger sequencing method. The frequency of each candidate was compared with that of control samples in both dominant and recessive models (Table 3). As a result, 6 out of 26 candidates had p-values less than 1.92 × 10$^{-3}$. Although all these variants were novel as AA or AT/ AU susceptibility variants, BTNL2 and HLA-A had previously been reported as genetic candidates for the development of AA [17, 28].

To determine whether there were any haplotypes composed within our candidate variants, we explored the LD structure among them by using genotype data from our AU patients (Table 4). As a result, we found 2 LD structures among 26 candidates; one included rs28362679, rs41559420, rs41542812, and rs1049107, whilst the other included rs116897146 and rs41542812 (p $= 0.019$, OR 4.0) did not reach the significance level of this study [17, 28].

Table 2. Sequencing summary of patient samples.

| Samples | Total reads | Uniquely aligned reads | % Covered bait bases$^1$ (≥1) | % Covered bait basesMean coverage depth in bait regions (x) |
|---------|-------------|------------------------|-----------------------------|---------------------------------------------------------|
| AU01    | 65,959,668  | 57,329,095             | 96.07                       | 87.25                                                   | 58.65                                                  |
| AU02    | 41,309,992  | 35,686,769             | 94.06                       | 79.73                                                   | 35.32                                                  |
| AU03    | 71,009,718  | 61,311,833             | 96.23                       | 87.72                                                   | 62.09                                                  |
| AU04    | 53,085,782  | 46,056,902             | 95.58                       | 84.9                                                    | 46.53                                                  |
| AU05    | 74,426,242  | 64,677,317             | 96.39                       | 88.34                                                   | 65.39                                                  |
| AU06    | 121,993,380 | 75,311,600             | 89.23                       | 76.22                                                   | 76.66                                                  |

$^1$Bases within the bait region defined as the region covered by more than one capture probe.

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Discussion

Our study revealed several immune-related susceptibility variants for AU and the LD between some of the most significant variants. Of the 2 statistically significant genes in the associated combination of alleles, BTNL2 has already been associated with the development of AA [17], while HLA-DRB5 has not previously been implicated in the pathogenesis of this disease. The HLA-DRB5 gene encodes the major histocompatibility complex, class II, DR beta 5. This class II molecule plays an important role in the immune system by presenting antigens in antigen-presenting cells.

The presence of a certain HLA gene might ensure more proficient presentation of a particular autoantigen and, consequently, the association of HLA genes with autoimmune diseases has received a great deal of attention. The associations of HLA-DRB5 with autoimmune disorders such as multiple sclerosis [29], systemic sclerosis [30], and rheumatic heart disease [31] have been reported. Given the assumed autoimmune development of AA, we hypothesized that HLA-DRB5 might also be responsible for the development of AA.

Although numerous previous studies including 2 genome-wide association studies (GWAS) have been performed, the association of HLA-DRB5 with AA has not yet been reported to our knowledge. In this study, however, the nonsynonymous SNP (rs41559420) in HLA-DRB5 was most significant in the comparative analysis of 20 AU patients with controls, showing the highest odds ratio among candidates (Table 3). This may indicate the existence of different pathological mechanisms between AU and AA, or these kinds of variants may have been missed, or not reported, in the previous studies because of their considerable rarity. Indeed, the Illumina 610 K and 550 K, used in the previous 2 GWAS studies performed on AA, do not contain this SNP [17, 18]. Even if these arrays did include it, it would not have been analyzed since SNPs with low minor allele frequency (MAF) are usually excluded during the quality control process (Table 3, see the allele frequencies in the columns of MAF).

In addition to BTNL2, we also replicated another gene, HLA-A, to be associated with AA at the gene level [28]. Besides these, other variants found in this study were all located in novel genes, including HLA-DMB, PMS2, and TLR1. The HLA-DMB gene encodes HLA class II beta chain paralogue like HLA-DRB5 [32] and this molecule is an important component of the adaptive immune system. TLR1, which plays fundamental roles in innate immunity [33, 34], was also first reported in AA. Since both adaptive and innate immunoregulatory genes are known to be...
involved in the pathogenesis of AA [17], these novel findings
warrant further research. Mutations in PMS2 are important in the
progression of human cancer [35,36], and deficiency of human
PMS2 is associated with impaired immunoglobulin class switch
recombination [37]. The association of cancer immunology
components with AA requires further validation.

In Table S4, we list all the pathogenic candidates satisfying the
candidate selection criteria of this study, regardless of gene
ontology, for the researchers interested in AA or AT/AU. Even if
only the immune-related variants were selected for this study,
there could be more explanations on disease development other
than autoimmunity, or unknown genes participating in the
immune system. Furthermore, as AA or AT/AU is a well-known
complex disorder determined by multiple genetic and environ-
mental factors, these listed variant would be open to a diversity of
interpretations when more patient samples are sequenced or
various studies are conducted on different points of view.

Genes involved in the hair development can be good examples
of such possible candidates for the AU pathogenesis, other than
immune-related genes. Previous studies have reported several
genes for hair development, such as HR, LHX2 [38], and genes
involved in Wnt signaling [39], Sonic hedgehog (Shh) signaling
[40], and bone morphogenetic protein (BMP) signaling pathways
[41]. Among the pathogenic candidates in Table S4, we found 3
variants in Wnt signaling genes, including AXIN2, POSTN, and

| Variant | Gene | Risk allele | p-value | OR | p-value | OR |
|---------|------|-------------|---------|----|---------|----|
| rs41559420 | HLA-DRB5 | A | <0.001 | 44.571 | <0.001 | - | 0.014 | 0.005 |
| rs28362679 | BTN2L2 | A | <0.001 | 30.214 | - | - | 0.021 | 0.007 |
| rs113337937 | HLA-A | del | <0.001 | 18.857 | - | - | - | 0.088 |
| rs116375983 | HLA-DMB | T | <0.001 | 8.917 | 0.159 | 3.861 | - | 0.14 |
| rs1805323 | PMS2 | T | 0.001 | 8.125 | 0.332 | 1.683 | 0.348 | 0.31 |
| rs117033348 | TLR1 | G | 0.001 | 7.754 | - | - | 0.014 | 0.032 |
| rs1126477 | LTF | T | 0.002 | 6.847 | 0.193 | 2.19 | 0.4 | 0.292 |
| rs2277244 | DMAT1 | T | 0.003 | 6.074 | - | - | 0.079 | 0.049 |
| rs2297950 | CHI1T | T | 0.006 | 4.024 | 0.017 | 7.667 | 0.295 | 0.174 |
| rs17130745 | GBP4 | C | 0.009 | 6.083 | 0.115 | - | 0.033 | 0.026 |
| rs78108426 | CIITA | A | 0.009 | 4.667 | - | 0 | 0.131 | 0.069 |
| rs41542812 | HLA-DQB1 | G | 0.015 | 5.4 | 0.01 | - | 0.058 | 0.037 |
| rs75268750 | CS | A | 0.019 | 4 | - | - | 0.072 | 0.048 |
| rs161704 | IL31RA | A | 0.023 | 3.16 | 0.18 | 2.156 | 0.358 | 0.295 |
| rs2276872 | CD96 | C | 0.024 | 3.025 | 0.387 | 2.667 | 0.157 | 0.116 |
| rs17847215 | INPP1 | C | 0.036 | 4 | - | - | 0.075 | 0.048 |
| rs2273346 | MASP2 | G | 0.046 | 2.522 | 0.081 | 4.059 | 0.175 | 0.184 |
| rs2730407 | DEF1 | T | 0.053 | 5.153 | - | - | 0.01 | 0.017 |
| rs4304840 | CLEC4D | G | 0.06 | 2.413 | - | 0 | 0.154 | 0.14 |
| rs166206892 | MICA | C | 0.084 | 2.227 | 0.069 | 4.553 | - | 0.153 |
| rs34531670 | KIR3DP1 | C | 0.106 | 2.114 | 0.248 | 2.593 | - | 0.14 |
| rs4911290 | C20orf185 | A | 0.223 | 3.2 | 0.494 | 1.143 | 0.248 | 0.288 |
| rs116897146 | KIR3DP1 | C | 0.226 | 1.66 | 0.541 | 1.495 | - | 0.139 |
| rs1799945 | HFE | G | 0.289 | 2.254 | - | - | 0.024 | 0.023 |
| rs1049107 | HLA-DQ81 | T | 0.3 | 1.548 | 0.094 | 3.625 | - | 0.161 |
| rs17611 | CS | T | 0.393 | 1.404 | 0.024 | 2.949 | 0.418 | 0.497 |

Abbreviations: OR, odds ratio; SNP, single nucleotide polymorphism; MAF, minor allele frequency; del, deletion; NGS, next-generation sequencing.

1Minor allele frequency of each variant in 1000 Genomes Phase I [27].
2Minor allele frequency of each variant in 155 controls used in this study.

Table 4. Linkage disequilibrium estimation among candidates (r2>0.3).

| Chromosome | Variant 1 | Variant 2 | r2 | D' |
|------------|-----------|-----------|----|----|
| 6          | rs28362679 | rs41559420 | 0.41 | 1 |
|            | rs41542812 | rs1049107 | 0.61 | 1 |
| rs41559420 | rs41542812 | rs1049107 | 0.68 | 1 |
| rs1049107  | rs41542812 | rs1049107 | 0.49 | 0.74 |
| rs41542812 | rs1049107 | rs41542812 | 0.77 | 1 |
| 19         | rs116897146 | rs34531670 | 0.75 | 1 |

Abbreviations: r2, squared correlation coefficient; D', Hardy-Weinberg equilibrium.
Novel Candidate Variants of Alopecia Universalis

Through next-generation sequencing technologies, it is predicted that these variants in complex traits [44]. Now, due to the advance of high-throughput technologies, it is expected that these approaches will potentially replace array-based technologies and open up a new era of genetic research [45]. The current findings are another example of the potential use of sequencing technologies to identify causative candidate genes in complex disorders.

In conclusion, through an exomic sequence analysis on immune-related genes, we found several candidate variants and haplotypes associated with AU. This study provides further evidence for the involvement of immune-related genes in the development of AA and identifies novel candidates such as HLA-DRB5, which may play a previously unrecognized role in the pathogenesis of the disease.

Supporting Information

Figure S1 The flow of filtration steps used for candidate variant selection. (PPTX)

Table S1 Demographic data and clinical presentations of study subjects. (DOC)

Table S2 Base coverage and Q-score of each variant in 155 controls and 6 cases. (XLS)

Table S3 Primer pairs for genotyping of candidate variants. (XLS)

Table S4 667 pathogenic candidates regardless of gene ontology. (XLS)

Table S5 Statistical results of 26 candidates in three different analyses. (XLS)

Author Contributions

Conceived and designed the experiments: JK OSK. Performed the experiments: SL SC. Analyzed the data: SL SHP HK. Contributed reagents/materials/analysis tools: HCE JS JK OSK. Wrote the paper: SL SHP HHR SJ.

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