Complement C5 Gene Confers Risk for Acute Anterior Uveitis

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Submitted: February 9, 2015
Accepted: May 20, 2015

PURPOSE. Polymorphisms in the genes encoding C3 and C5 are associated with several immune-mediated diseases. However, the association of C3 and C5 SNPs with acute anterior uveitis (AAU) has not yet been investigated and was the purpose of the study described.

METHODS. Genotyping was performed for six SNPs in C3 and four SNPs in C5 in 395 AAU patients with ankylosing spondylitis (AS), 397 AAU patients without AS, and 597 healthy controls by PCR-restriction fragment length polymorphism (PCR-RFLP) or TaqMan SNP assay. The mRNA expression was detected by real-time PCR. Cytokine production and total C5 serum concentrations were measured by ELISA.

RESULTS. The frequency of the GG genotype of rs2269067 in C5 was increased in AAU patients with or without AS compared to controls (Pc = 4.0 × 10⁻³, odds ratio [OR] = 1.94 and Pc = 9.4 × 10⁻⁵, OR = 1.89, respectively). In addition, mRNA and serum concentrations of C5 were significantly increased in rs2269067 GG cases as compared to that in CG or CC cases (P = 0.012, P = 0.002; P = 0.021, P = 0.006, respectively). An increased production of interleukin-17 was observed in rs2269067 GG cases compared to CG or CC cases (P = 5.1 × 10⁻⁴, P = 1.4 × 10⁻⁴, respectively).

CONCLUSIONS. The C5 rs2269067 GG genotype confers risk for AAU in a Chinese population and is associated with an elevated C5 serum concentration and an increased IL-17 production.

Keywords: acute anterior uveitis, ankylosing spondylitis, C3, C5

Uveitis is one of the devastating ocular diseases causing blindness.¹ Acute anterior uveitis (AAU), including iritis or iridocyclitis, shows an acute inflammation onset occurring in the anterior segment of the eye which can lead to visual impairment.² Ankylosing spondylitis (AS) is a chronic inflammatory disease that involves the spine and other extra-articular organs, such as the eye, skin, and cardiovascular system.³ Acute anterior uveitis is thought to be the most frequent extra-articular manifestation of AS.⁴ Previous studies showed that nearly half of patients with HLA-B27-positive AS will experience a bout of AAU during their disease course.⁵ Recently, many studies have shown that, besides HLA-B27, many other genetic factors are involved in the onset of AAU and AS, such as CTLA-4, PTPN22, and TNF in AAU,⁶,⁷ and ERAP1 and IL-23R in AS.⁸

The complement system has an important role in the innate immune system.⁹ Previous studies revealed that certain genetic variants of C3 and C5 are a risk factor for several autoimmune disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).¹⁰–¹⁵ An association between C3 and C5 polymorphisms in ocular disease has been investigated in age-related macular degeneration.¹⁶–¹⁸ Furthermore, several studies have demonstrated that the complement system has a critical role in the pathogenesis of experimental autoimmune anterior uveitis (EAAU) or endotoxin-induced uveitis (EIU).¹⁹–²¹ Although the exact pathogenesis of AS remains unknown, previous studies have shown that complement components are involved in the pathogenesis of AS.²²–²⁴ However, to our knowledge the association between C3 and C5 genetic variants has not yet been addressed in other ocular diseases, such as uveitis and, therefore, was the subject of the study described above.

MATERIALS AND METHODS

Study Design

A total of 395 AAU patients with AS, 397 AAU patients without AS, and 597 normal controls were recruited. All the participants were Chinese Han. Blood samples were taken from patients and controls visiting the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) or the Zhongshan Ophthalmic Center of Sun Yat-sen University (Guangzhou, China). The diagnosis of AS was based on the modified New York criteria.²⁵ Inclusion of AAU patients was based on the anterior uveitis, characterized by sudden onset and with limited duration of less than 3 months. Diagnosis was based on clinical features, including ciliary injection, dust-like keratic precipitates, a large number of cells in the anterior chamber, and obvious aqueous flare. Typically, these patients showed recurrent episodes after first disease onset. There was no visible retinal or choroid lesion except a reactive and transient papilledema or macular edema.²⁶,²⁷ Patients were excluded from this study if they suffered from other uveitis entities or if the diagnosis was not definitely made. The study was authorized by the First Affiliated Hospital of Chongqing Medical...
University Ethics Research Committee (Permit Number, 2009-201008). All procedures in this study were based on the principles of the Declaration of Helsinki. Before accepting the samples of peripheral venous blood (EDTA-anticoagulated whole blood or serum), all the participants needed to provide written informed consent. Serum was separated at 4000 rpm for 30 min and stored at −80°C.

**Single Nucleotide Polymorphism (SNP) Selection**

The selection of C3 and C5 SNPs was based on previous studies. Nine SNPs of C3, including rs7951, rs344555, rs2230199, rs1047286, rs2250656, rs3745568, rs2241394, and rs14789257, were analyzed. Among them, rs2230199, rs1047286, and rs14789257 were monozygous in the Han Chinese population (1000 genome database) and, therefore, were not included in our study. Two pairs of SNPs (rs3745568 and rs408290, rs171094 and rs2250656) are in linkage disequilibrium (D = 0.91, r² = 0.45 and D = 1, r² = 0.13, respectively). Two SNPs, including rs408290 and rs422001, were selected as TagSNPs. Finally, six C3 SNPs, including rs408290, rs7951, rs344555, rs2250656, rs423001, and rs2241394, were selected for this study. Ten SNPs of C5 (rs2269067, rs17611, rs7026551, rs10985126, rs7037673, rs1468673, rs7040033, rs2269066, rs25681, rs7027797) were analyzed as candidates for our study and, among these, rs7026551, rs2269066, rs25681, rs10985126, rs7037673, rs1468673, and rs2269067 are in linkage disequilibrium (r² ≥ 0.80). Also, rs7037673, rs17611, rs25681, and rs7040033 are in strong linkage disequilibrium (r² = 1). Single nucleotide polymorphism rs1017119 was selected as a TagSNP. Finally, four C5 SNPs, including rs2269067, rs7040033, rs1017119, and rs7027797, were selected for this study. Taken together, 10 SNPs were analyzed for this study (six C3 SNPs and four C5 SNPs).

**DNA Extraction and SNP Genotyping**

Genomic DNA was extracted from peripheral blood with the Qiagen DNA Mini blood kit. Two SNPs, including rs422001 and rs2241394, were genotyped by PCR assay (TaqMan assay ID, C_987748_10, respectively) on real-time PCR (Applied Biosystems, Carlsbad, CA, USA). The other eight SNPs were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) method with proper primers and restriction enzymes (the primer sequences and restriction enzymes are shown in Supplementary Table S1). Four percent agarose gels were used to separate the digestion products and colored with GoldView (SBS Genetech, Beijing, China). Data were analyzed to ensure that our study population was in Hardy-Weinberg equilibrium. Ten percent of study samples were used randomly for direct sequencing to assure the validity of the SNP genotyping.

**Cell Isolation and Culture**

Peripheral blood mononuclear cells (PBMCs) from healthy genotyped individuals were isolated using Ficoll-Hypaque density-gradient centrifugation. The isolated PBMCs were seeded in 24-well plates (2 × 10⁶ cells per well) and cultured for 1 or 3 days in 100 μg/mL streptomycin, RPMI medium 1640 supplemented with 10% fetal calf serum (FCS, Belgium) and 100 U/mL penicillin. Peripheral blood mononuclear cells were stimulated with a mixture of anti-CD3 (5 μg/mL; eBioscience, San Diego, CA, USA) and anti-CD28 antibodies (1 μg/mL; eBioscience) for 72 hours, then the production of IL-17, IFN-γ, and IL-10 was measured in the culture supernatants. To measure the level of IL-1β, IL-8, TNF-α, IL-6, and MCP-1 following an inflammatory stimulus, we cultured PBMCs with 100 ng/mL lipopolysaccharide (100 ng/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) for 24 hours and then collected supernatants for cytokine measurements.

**Real-Time PCR**

TRIzol (Invitrogen, Carlsbad, CA, USA) and transcriptase kit (Applied Biosystems) were used for total RNA extraction and cDNA reverse transcription, respectively. The primer sequences for C3 and C5 were as follows: forward, 5′-GGGACAGCTC AAGGTTCTAC GC-3′; reverse, 5′-CAAGGTCTCATCAGGCCGAC AG-3′; and forward, 5′-GGCACAAT GCCTCTCAAAATG-3′; reverse, 5′-CCCCACCA AGTCCCTCAGTA-3′, respectively. β-Actin was selected as the internal reference gene and the 2ΔΔCt method was used to calculate the relative expression for each gene.

**ELISA Assay**

The human Duoset ELISA development kit (R&D Systems, Minneapolis, MN, USA) was used to measure the C5 concentration in serum and the concentration of IL-17, IFN-γ, IL-10, IL-8, TNF-α, IL-6, IL-β, and MCP-1 in PBMC culture supernatants according to the manufacturer’s protocols.

**Statistical Analysis**

To analyze the frequencies of genotypes and alleles, the χ² test (SPSS version 17.0; SPSS, Inc., Chicago, IL, USA) was applied to compare the differences between patients and controls. The mRNA expression, C5 serum concentrations and various cytokines were analyzed by 1-way ANOVA or a nonparametric test for independent samples among three genotype cases. Bonferroni correction was used for multiple comparisons. A total of 10 SNPs, including 20 alleles and 27 genotypes, were analyzed in this study. As the two alleles of one SNP showed the same result, the P values of allele comparisons were corrected by a factor 10. Similarly, there were 27 different genotypes of all the studied SNPs, so the P values for the frequency of genotypes was corrected by a factor 27. Significance was tested by 1-tailed P values < 0.05 (SPSS version 17.0).

**RESULTS**

**Clinical Characteristics of AAU Patients With or Without AS**

The details of clinical features, age, and sex distribution in the AAU patient group and controls are shown in Supplementary Table S2.

**Increased Frequency of C5 GG Genotypes in AAU Patients**

The 10 SNPs were genotyped in 395 AAU patients with AS, 397 AAU patients without AS, and 597 normal controls. The genotype frequencies of the 10 SNPs did not deviate from HWE in normal controls (P > 0.05). The results showed that the frequencies of the C5 rs2269067 GG genotype and G allele in AAU patients with AS (AAU+ AS−) was significantly increased compared to controls (Pc = 4.0 × 10⁻⁵, odds ratio [OR] = 1.94 and Pc = 1.3 × 10⁻⁴, OR = 1.66, respectively). Similar to AAU+ AS−, the frequencies of GG genotypes and G alleles of rs2269067 in AAU+ AS− patients were also higher than that in controls (Table 1). The genotype and allele frequencies of the other three SNPs of C5 and the six SNPs of C3 were not
Increased C5 mRNA Expression and Serum C5 Protein Levels in Individuals With rs2269067 GG Genotype

The aforementioned results revealed an obvious association of C5 rs2269067 with AAU. We next investigated mRNA expression of C5 in PBMCs from healthy individuals with a known SNP genotype. The mRNA expression of C5 rs2269067 in GG cases was significantly increased compared to CC and CG cases (P = 0.002, P = 0.012, respectively; Fig. 1).

A separate set of healthy genotyped individuals was tested for their circulating C5 serum protein level. The C5 serum concentration also was elevated in GG cases compared to that in CC and CG cases (P = 0.006, P = 0.021, respectively; Fig. 1).

The Influence of C5 rs2269067 on Cytokine Production

A further experiment was performed to examine whether different genotypes of C5 affected cytokine production. The level of IL-17, IFN-γ, TNF-α, IL-10, IL-1β, MCP-1, IL-6, and IL-8 was tested in the culture supernatants of stimulated PBMCs from genotyped healthy controls. The results showed that the production of IL-17 in C5 rs2269067 GG cases was higher than that in CC and CG cases (P = 5.1 × 10⁻³, P = 1.4 × 10⁻⁴, respectively; Fig. 2). The production of the other seven cytokines was not associated with C5 genotype.

Discussion

In the present study, we investigated the association of six C3 SNPs and four C5 SNPs with AAU in a Han Chinese population. The results showed that C5 rs2269067 was a risk factor for AAU. Functional studies showed that C5 mRNA and protein expression in the C5 GG genotype cases were increased compared to the other two genotype cases. Additionally, the production of IL-17 was significantly increased in the C5 rs2269067 GG genotype cases.

The complement system has been considered as an important element in the innate as well as in the adaptive immune response. Other studies have demonstrated that the blockade of the complement alternative pathway could suppress EAAU via a modulation of the T cell response. Blocking the formation of the C3 convertase, resulted in the inhibition of EAAU by reducing several cytokines, such as IFN-γ, IP-10, and ICAM-1, in a Lewis rats model. In the present study, we did not find evidence for an involvement of C3 polymorphisms in the risk for AAU development. This may be due to differences between the animal models of uveitis and human AAU.

Recent studies showed that suppression of C5 could protect against tissue damage and reduce disease severity during the autoimmune response in mouse models of EAU. Bora et al. found that EAAU could be induced by antigen-specific CD4⁺ T cells directed against antigens derived from iris and ciliary body in a Lewis rats model. Of interest is that a significantly increased expression of CD4⁺IL-17⁺Th17 cells and CD4⁺IFN-γ⁺Th1 cells was found in the peripheral blood of patients with HLA-B27-positive AAU. These results suggested that C5 activation in combination with a CD4⁺ T cell response, including the Th17 cell and Th1 cell subsets, are both critical for the development of EAAU.
Only few clinical studies have shown an association between complement C5 with AAU. Complement C5-derived chemotactic activity was present in inflamed aqueous humor in an endotoxin-induced rabbit model of AAU and the chemotactic activity could be inhibited by antibodies to C5.21 This was confirmed in a clinical study whereby it was shown that the levels of C5a in inflamed aqueous humor from patients with AAU was significantly higher than that in normal controls or patients without inflamed aqueous humor.42

Only few studies have shown an association between C5 polymorphisms with autoimmune or autoinflammatory disorders. Chang et al.15 found that C5 rs2269067 was weakly associated with RA (OR: 1.27). To our knowledge the association between genetic variants of C5 and uveitis has not yet been reported.

Genetic studies in mice have shown that C5 variants have an important role in the pathogenesis of a collagen induced arthritis model.43–45

How C5 SNP rs2269067 influences the risk for AAU is not yet clear. Preliminary data showed that the C5 mRNA expression was enhanced in the rs2269067 GG genotype cases. We furthermore showed that C5 concentration in serum was elevated in rs2269067 GG genotype cases. Liu et al.46 found that C5a could promote the production of IL-17 by human T cells in AMD patients. Of interest was our finding that the production of IL-17 by stimulated PBMCs was increased in the GG genotype cases. It is possible that the C5 GG rs2269067 genotype may increase the production of IL-17 through C5 serum protein, thereby influencing the development of AAU. This is a speculative hypothesis and further studies are needed to examine the exact role of C5 genotypes and IL-17 production in the pathogenesis of AAU. Therefore, whether modulation of C5 could have therapeutic implications in our AAU patients also deserves further basic investigation. Of interest is that anti-C5 therapy has been shown to reduce the severity of disease in mouse models of EAU.38
FIGURE 2. The influence of C5 rs2269067 on cytokine production. Cytokine production (a–h) by anti-CD3/CD28 (a, e, h) or LPS (b–d, f, g) stimulated PBMCs from healthy controls with a known C5 rs2269067 genotype (GG, n = 13–20; CG, n = 13–21; CC, n = 11–14). Significance was examined by two independent samples nonparametric test or a 1-way ANOVA test. Data are shown as mean ± SD.
Some limitations in our study should be taken into account. This study was performed only in a Chinese Han population, and replication in an independent sample would strengthen our findings. Our findings also must be confirmed in other ethnic populations. All patients used in our study were recruited from an ophthalmology department, only including uveitis patients. Whether the association we found also exists between the C5 genotypes and AS patients without uveitis remains unclear and must be further studied. We studied only six C3 SNPs and four C5 SNPs, and cannot exclude a possible association with other as yet unknown SNPs. The effect of C5 genotypes on C5 and IL17 expression was performed in healthy genotyped individuals and not in patients, to exclude a confounding effect of the inflammatory response or installed therapy. Future studies could be planned to investigate how disease activity affects the C5 expression in genotyped patients. It should be noted that the C5 mRNA expression in the GG genotype individuals showed a large variability in this study for which we do not yet have an explanation. Healthy individuals were nonselectively collected for C5 mRNA examination over a period ranging from November 2014 to January 2015 and did not suffer from eye diseases or any immune-related disorder.

A variety of cells can produce IL-17, such as Th17 cells, NK cells, gamma/delta T cells, macrophages, and neutrophils.47–52 We only studied IL-17 production in a total PBMC sample and further subset analysis is necessary to investigate which cell type is responsible for the augmented IL17 response in individuals with a certain C5 genotype.

In conclusion, our results revealed that the C5 rs2269067 confers a risk for AAU in a Chinese Han population. The risk genotype affects the production of C5 and of proinflammatory cytokines, such as IL-17.

Acknowledgments

The authors thank all the participants in this study.

Supported by Natural Science Foundation Major International (Regional) Joint Research Project (81320108009), Key Project of Natural Science Foundation (81130019), National Natural Science Foundation Project (31370893, 81270990), Basic Research Program of Chongqing, Specialties Construction Program of China, and Fund for PAR-EU Scholars Program.

Disclosure: D. Xu, None; S. Hou, None; Y. Jiang, None; J. Zhang, None; S. Cao, None; D. Zhang, None; L. Luo, None; A. Kijlstra, None; P. Yang, None

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