Association of Long Noncoding RNAs Polymorphisms With Ankylosing Spondylitis, Vogt-Koyanagi-Harada Disease, and Behcet’s Disease

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PURPOSE. Long noncoding RNAs (lncRNAs) are emerging as important regulators of inflammatory immune responses, whereby genetic variants may affect this biologic function. This study aimed to investigate the association of 110 single nucleotide polymorphisms (SNPs) of lncRNAs, known to be associated with autoimmune disease, in patients with ocular Vogt-Koyanagi-Harada (VKH) disease, Behcet’s disease (BD), and acute anterior uveitis (AAU) with or without ankylosing spondylitis (AS).

METHODS. A two-stage case-control study was performed on 1626 VKH patients, 384 BD patients, 624 AAU with AS, 751 AAU without AS, 720 AS without AAU, and 3350 healthy subjects. lncRNAs 110 SNPs were genotyped using MassARRAY System or TaqMan SNP assays. The gene expression and cytokine production were measured using real-time PCR or ELISA.

RESULTS. The frequency of the C allele of rs4937362 in RP11-264E20.1 was markedly decreased in the AS without AAU group compared with controls (Combined P = 9.37 x 10^-7, odds ratio [OR] = 0.73). An increased frequency of the A allele of rs6871626 between UBLCP1, IL12B, and LOC285627 was found in VKH patients compared with controls (Combined P = 1.88 x 10^-6, OR = 1.19). UBLCP1, IL12B, and LOC285627 were expressed in human uveal tissues. Functional studies showed a decreased LOC285627 mRNA expression in peripheral blood mononuclear cells (PBMCs) and an increased IL-10 production in PBMCs following LPS stimulation in rs6871626 CC genotype carriers.

CONCLUSIONS. Our study is the first to show that rs4937362/RP11-264E20.1 is associated with AS and that rs6871626 is associated with VKH disease in Chinese Han. The protective rs6871626 genotype was shown to regulate the expression of LOC285627 and to increase the production of the anti-inflammatory cytokine IL-10.

Keywords: Behcet’s disease, VKH disease, acute anterior uveitis, ankylosing spondylitis, lncRNAs, disease susceptibility

Uveitis is an inflammation of the uveal tract and can lead to vision loss. According to the anatomic location, uveitis can be divided into panuveitis, anterior uveitis, intermediate uveitis, and posterior uveitis.1急性 anterior uveitis (AAU) is a sudden-onset uveitis and mostly presents in a unilateral fashion. Almost 50% of anterior uveitis patients carry the human leukocyte antigen B27 (HLA-B27).2 Ankylosing spondylitis (AS) is an inflammatory disease that also shows a strong association with HLA-B27.3 Approximately one-third of HLA-B27-positive AS patients also suffer from AAU.4 In addition to AAU, Vogt-Koyanagi-Harada (VKH) disease and Behcet’s disease (BD) are the two most general uveitis entities seen in China.5 BD is an inflammatory disease accompanied by the following manifestations: oral aphthae, skin lesions, and genital ulcers. VKH disease is an autoimmune disease characterized by a bilateral granulomatous uveitis that is often associated with systemic symptoms, including dysacusis, alopecia, poliosis, and vitiligo.6

The exact etiology and pathogenesis of these diseases remain unclear. Many studies have shown that genetic factors and the dysfunction of immune responses play an important role in these diseases. HLA-DR4 and HLA-DRw53 are associated with VKH disease, and HLA-B51 is associated with BD.7–9 Additionally, many non-HLA genes, including IL17E, IL-23A, TNEAIP3, C3, IL-12B, MIF, TRAF5, and TRAF3IP2, are associated with both BD and VKH disease.10 An abnormal immune response is also involved in these three diseases. The imbalance in T-cell homeostasis, including cytotoxic T cells, Th1 cells, Treg cells, and Th17 cells, have been implicated in the pathogenesis of both BD and VKH disease.10,11 Genome-wide association studies (GWAS) showed that loci at 1p31.2 and 10q21.3 were associated with the susceptibility to VKH disease. GWAS also identified the variants in the IL10, MHC class I, and IL23R/IL12RB2 regions were associated with the development of BD.12–14 Both AAU and AS are strongly associated with HLA-
B27 as well as with a variety on non-HLA genes, such as FOXO1, GATA-3, and FOXP3. However, some of the associated regions were located on intergenic regions, suggesting that noncoding RNAs (ncRNAs) may be involved in the development of these diseases.

The mammalian genome has a large number of RNAs that do not encode proteins, which are called ncRNAs. According to the transcript size, ncRNAs can be divided into many types, including microRNAs (miRNAs) and long ncRNAs (lncRNAs). miRNAs range from 19 to 25 nucleotides, and have shown to play important roles in various human immune diseases, including VKH disease, BD, AU, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), systemic sclerosis, and primary Sjögren’s syndrome. IL-12, which have all been implicated in the pathogenesis of a variety of uveitis entities. Variations of lncRNAs have an important role in various biological and physiological processes, including transcription, chromatin modification, and posttranscriptional processing.

A number of studies have suggested that lncRNAs are also associated with the susceptibility to autoimmune disease, such as SLE, RA, and MS, were shown to be associated with the susceptibility to AU. lncRNAs are a group of noncoding potential transcripts that are longer than 200 nucleotides. Over the past few decades, lncRNAs have been considered as transcriptional “noise.” Recent studies have, however, shown that lncRNAs have an important role in various biological and physiological processes, including transcription, chromatin modification, and posttranscriptional processing. Number of studies have suggested that lncRNAs are also involved in the development of autoimmune disease. lncRNAs can regulate the development of various immune cells, such as B and T lymphocytes, macrophages, and dendritic cells (DCs).

A number of studies have shown that the pathogenesis of autoimmune diseases, such as SLE, RA, and MS, were associated with lncRNAs. lncRNAs are especially expressed in cells responsible for the innate and adaptive immune response. Examples include LncRNA-CD244, lincRNA-Cox2, THRIL, Th2-1CR, and Inc-DC, which have been shown to be expressed in T cells, macrophages, and DCs. These lncRNAs regulate the transcription of immune-response genes and the production of related cytokines, such as IFN-γ, TNF-α, IL-13, IL-5, IL-4, and IL-12, which have all been implicated in the pathogenesis of a variety of uveitis entities.

A number of studies have shown that the pathogenesis of autoimmune diseases such as SLE, RA, and MS, were associated with lncRNAs. Lnc-USP50-2, Lnc-ZNF354A-1, Lnc-ERG2C3, and Lnc-LIN54-1 have been shown to be involved in the development of AS.

Up to now, several lncRNAs have been found to be implicated with ocular disorders, such as ocular tumors, glaucoma, diabetic retinopathy, proliferative vitreoretinopathy, and corneal vascularization. As mentioned above, lncRNAs play a role in inflammation, and this regulatory control may be affected by genetic variants, which in turn might explain why a certain variant predisposes or protects against immune disease. Up to now, the relationship between lncRNAs and uveitis has yet to be reported, and the current study was therefore performed to address this issue. The results showed that rs6871620 was associated with VKH disease and SNP rs4037362 in RP11-264E20.1 with the group of AS patients without AU. Preliminary experiments were performed to investigate the biological function of lncRNA genotypes.

Materials and Methods

Study Population

A total of 1626 VKH patients, 384 patients with BD, 624 AAU+AS patients, 751 AAU−AS patients, 720 AS+AAU− patients, and 3305 unrelated healthy controls were recruited from The First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All patients and controls were Chinese Han. The study was performed in two stages, whereby patients and controls for the first stage came from Southwest China, including Chongqing and Sichuan. These included patients with BD (n = 384), VKH (n = 384), AAU with AS (n = 384), AAU without AS (n = 384), and AS without AAU (n = 384), and healthy controls (n = 768). A confirmatory second-stage study was performed with a cohort of patients coming from North China (Hebei, Beijing), Central China (Henan, Hubei), and East China (Anhui, Shandong, and Zhejiang). This group included 1242 VKH patients, 240 AAU with AS patients, 367 AAU without AS patients, 336 AS without AAU patients, and 2557 controls. The nonparametric Mann-Whitney U test or χ2 test was used to compare the differences of the clinical characteristics between the first and second disease groups. However, there was no difference between the first- and the second-stage groups (P > 0.05).

All uveitis patients were seen at the Department of Ophthalmology, and AS patients were seen by our rheumatology department. The diagnosis of VKH disease and BD was made strictly according to the International Study Group’s criteria for VKH disease and BD, respectively. The diagnosis of AAU was made according to the clinical features, including eye pain, the loss of vision, ciliary congestion, keratic precipitates, anterior chamber flare, iris abnormalities, and the appearance of inflammatory cells in the anterior chamber. Additionally, AAU patients show a sudden onset, as well as recurrent episodes, with a duration of no more than 3 months. Optical coherence tomography and ultrasound biomicroscopy were helpful in the diagnosis of AAU. The diagnosis of AS was made according to the 1984 modified criteria of New York. HLA-B27 was tested in 83% of the AS patients. All participants enrolled in this study signed a written informed consent voluntarily. All procedures of this study followed the principles of the Declaration of Helsinki and were approved by the ethical committee of Chongqing Medical University (Permit Number: 2009-201008).

The Selection of Single Nucleotide Polymorphisms (SNPs)

The selection of lncRNAs SNPs was based on three databases, including SNP@lnicTFBS (http://210.46.85.180:8080/SNP_lnic_lnc_tfbs/, in the public domain), lncRNAsNP-human (http://bioinfo.life.hust.edu.cn/lncRNAsNP/, in the public domain), and lncSNP (http://210.46.80.146/lincsnp/, in the public domain). Finally, 110 SNPs, shown previously to be associated with autoimmune disease, were selected for this study (Supplementary Table S1). Linkage disequilibrium was tested between SNPs in case a positive association was found.

The Extraction of DNA and Genotyping

Genomic DNA from peripheral blood samples was extracted by the QIAamp DNA Mini blood kit (QIAGEN, Valencia, CA, USA) based on the manufacturer’s protocols. The extracted DNA was diluted and then stored at −80°C until used.

The genotypes of lncRNAs SNPs were examined by MassARRAY System (Sequenom, San Diego, CA, USA) or TaqMan SNP assays. The primers used to genotype were made according to MassARRAY Assay design software. All SNPs were genotyped using Sequenom MassARRAY system (Sequenom) based on the manufacturer’s manuals in the first stage of our study except rs1991866. Rs1991866 was genotyped by TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 7500 Real-Time PCR.
system according to the manufacturer's manuals. In the second-stage study, selected SNPs, including rs6871626, rs793108, rs4937362, rs73013527, and rs2156698, were genotyped by TaqMan SNP Genotyping Assay according to the manufacturer's manuals. The results of genotyping assay data were calculated by TYPER software version 4.0 (Sequenom, San Diego, CA, USA) or TaqMan Genotyper Software.

**Cell Preparation, RNA Extraction, and Real-Time Quantitative PCR**

Fresh venous blood of healthy subjects was used to isolate peripheral blood mononuclear cells (PBMCs) using Ficoll-Hypaque density gradient centrifugation. Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The Takara transcriptase kit (Takara, Dalian, China) was used to detect the mRNA expression of IL12B, UBLCP1, LOC285627, ETS1, FLI1, and RP11-264E20.1 by real-time quantitative PCR with the ABI 7500 System (Applied Biosystems, Warrington, UK) in accordance with the manufacturer's manuals. Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) was used to detect the mRNA expression of IL12B, UBLCP1, LOC285627, ETS1, FLI1, and RP11-264E20.1 by real-time quantitative PCR with the ABI 7500 System (Applied Biosystems). The primers of the forward and reverse were as follows: IL12B: 5'-GCTGTTGAGGGCTGTC-3' and 5'-ACCAGCAGGTGAAAGCTC-3'; UBLCP1: 5'-TCTCGCCAGTGAAAGTGTACATA-3' and 5'-CTCCTGCAAAGACCTGTGTC-3'; ETS1: 5'-GCAGCCAGTCATCTTTCAACAGCC-3' and 5'-CGATCCACACGGAGTACTTG-3'; LOC285627: 5'-TCACGAGCTCATTTCTCAAGGCG-3' and 5'-CGATCCACACGGAGTACTTG-3'; FLI1: 5'-TCTACATTGCTCCCACCACTC-3' and 5'-CAAGCGCAAAGGGATGT-3'; RP11-264E20.1: 5'-TGTGACTTGGAGGGCTGT-3' and 5'-CAAGGGCAAGGGAGATGT-3'. Relative expression levels of genes were calculated using the 2^ΔΔCt method.

**Cell Culture and ELISA**

PBMCs isolated from peripheral blood were seeded at 1 x 10^6 cells per well in 24-well plates in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The supernatants obtained after 24 hours of lipopolysaccharide (LPS) stimulation (100 µg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) were tested for the concentration of a number of cytokines, including IL-10, IL-1β, IL-17, IL-8, IFN-γ, TNF-α, IL-6, and MCP-1 using human Duoset ELISA development kits (R&D Systems, Minneapolis, MN, USA).

**The Expression of IL12B, LOC285627, and UBLCP1 in the Human Uveal Tissues**

Human ocular tissue, including iris, ciliary body, and choroid, was dissected from a Han Chinese donor eye (male, 19 years old, died of leucocythemia) and TRIZol reagent was used to extract RNA. The mRNA expression of UBLCP1, IL12B, and LOC285627 was examined according to the manufacturer's manuals. Amplified production was stained by GoldView (SBS Genetech Beijing, China) and visualized on 3% agarose gels using a camera system (ChemiDoc XRS, Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

The Hardy-Weinberg equilibrium in healthy subjects of all detected SNPs was tested by SHEsis software (Shanghai Jiao Tong University, Shanghai, China). The χ^2 test was used to compare the differences of genotype or allele frequency between patients and healthy controls using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). The obtained P value was adjusted using Bonferroni correction according to the number of analyses. P < 0.05/110 = 4.55 x 10^-4 was considered as statistically significant. The nonparametric Mann-Whitney U test or independent samples T test was used to compare the expression of IL12B, UBLCP1, ETS1, FLI1, LOC285627, RP11-264E20.1, and cytokine production (IL-10, IL-1β, IL-17, IL-8, IFN-γ, TNF-α, IL-6, MCP-1) in the various genotype groups.

**RESULTS**

**Clinical Characteristics of Participating Uveitis and AS Patients**

The details of the clinical characteristics, age, and gender of the participants included for the two stages of the study are shown in Table 1. No statistical difference was observed concerning disease manifestations between the two groups.

**The Frequencies of Genotypes and Alleles of Involved IncRNAs SNPs in the First-Phase Study**

The frequencies of genotypes and alleles of 110 SNPs of IncRNAs were analyzed in the first-stage discovery cohort that included patients with BD (n = 384), VKH (n = 384), AAU with AS (n = 384), AAU without AS (n = 384), and AS without AAU (n = 384) and were compared with data obtained from healthy controls (n = 768) in the first-stage study. All participants for the first-stage study came from Southwest China, including Chongqing and Sichuan. The results showed a significantly increased frequency of the rs6871626 GA genotype, A allele, and a decreased frequency of the CC genotype (P = 6.04 x 10^-5, odds ratio [OR] = 1.66; P = 5.77 x 10^-5, OR = 1.45; P = 2.13 x 10^-6, OR = 0.54, respectively) in VKH patients as compared with controls (Table 2). We also found increased frequencies for the T allele of rs793108 (Supplementary Table S2), an increased frequency for the TT genotype, and a decreased frequency for the C allele of RP11-264E20.1/ rs4937362 in the AS without AAU group as compared with controls (P = 1.28 x 10^-7, OR = 1.42; P = 3.66 x 10^-5, OR = 1.69, P = 2.11 x 10^-5, OR = 0.67, respectively) (Table 3). No association was observed for the other patient groups tested (Supplementary Tables S3–S5).

**The Frequencies of Genotype and Allele of Involved IncRNAs SNPs in the Second and Combined Study**

To further verify the first-stage results, we performed a confirmatory study of the disease-associated SNPs rs6871626, rs793108, and rs4937362 using an independent cohort including 1242 VKH patients, 240 AAU with AS patients, 367 AAU without AS patients, 356 AS without AAU patients, and 2537 controls from North China (Hebei, Beijing), Central China (Henan, Hubei), and East China (Anhui, Shandong, and Zhejiang). The results confirmed that the frequency of the rs6871626 CC genotype was decreased and A allele was increased in VKH patients as compared with controls (P = 2.10 x 10^-2, OR = 0.85, P = 4.20 x 10^-2, OR = 1.11, respectively) (Table 2). Additionally, the frequencies of RP11-264E20.1/rs4937362 TT genotype was increased and C allele was decreased in the AS without AAU patients as compared with controls (P = 2.00 x 10^-3, OR = 1.45, P = 1.00 x 10^-3, OR = 0.74, respectively) (Table 3). The combined results showed an increased frequency in the
The percentage of HLA-B27 is the proportion of patients with AS without AAU who were tested for HLA-B27, so the percentage difference of both groups did not perform. The nonparametric Mann-Whitney U test or χ² test was used to compare the differences. However, there was no difference between the first- and the second-stage groups (P > 0.05).

RP11-264E20.1/rs4937362 TT genotype and a decreased frequency in the C allele in AS/AAU patients as compared with healthy controls (P = 2.29 × 10⁻⁶, OR = 1.49; P = 9.37 × 10⁻⁷, OR = 0.73, respectively) (Table 3). In addition, the results showed a decreased frequency of the rs6871626 CC genotype and an increased frequency of the A allele in VKH patients as compared with controls (P = 1.59 × 10⁻³, OR = 0.76; P = 1.88 × 10⁻¹⁴, OR = 1.19, respectively) (Table 2). To verify the association results of rs4937362, a linkage disequilibrium (LD) block was constructed using Haploview software (Whitehead Institute for Biomedical Research Cambridge, MA, USA) (Supplementary Fig. S1) and the other two SNPs rs2156698 and rs73013527 linked with rs4937362 (r² ≥ 0.89) were chosen to confirm the association. The results showed a consistent association of rs2156698 and rs73013527 with AS/AAU (P = 4.38 × 10⁻¹⁴, OR = 0.67, respectively) (Table 3). However, no association was found for SNP rs793108 (Supplementary Table S2).

The Effect of rs6871626, rs4937362 Genotypes on IL12B, UBLCP1, LOC285627, ETS1, FLII, and RP11-264E20.1

The aforementioned results showed the association of rs6871626 with VKH disease and rs4937362 with AS. Additional experiments were performed to elucidate the functional role of rs6871626 and rs4937362 on gene expression. Because immunosuppressive drug treatment may affect gene expression results of patients, normal genotyped controls were collected for the expression study. The results showed
TABLE 2. The Association of rs6871626 With VKH Disease

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<th>Genotype/Allele</th>
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<td>%</td>
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P value of the first and combined stage less than 0.05/110 = 4.55 × 10⁻⁵ was considered significant.

TABLE 3. The Association of rs4937362, rs2156698, and rs73013527 With AS<sup>+</sup>AU<sup>+</sup>

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P value of the first and combined stage less than 0.05/110 = 4.55 × 10⁻⁵ was considered significant.

<sup>*</sup> SNPs rs2156698 and rs73013527 linked with rs4937362 (r² = 0.89) were chosen to confirm the association between rs4937362 and AS.

**FIGURE 1.** The influence of diverse genotypes of rs6871626 on the expression of LOC285627 (CC: n = 30, AC+AA: n = 24), IL12B (CC: n = 15, AC+AA: n = 16), UBLCP1 (CC: n = 18, AC+AA: n = 20), in PBMCs of healthy individuals. The mRNA expression levels of LOC285627 (a), IL12B (b), and UBLCP1 (c) were examined in PBMCs of healthy individuals with a variety of genotypes of rs6871626.
The effect of rs6871626 on cytokine production. PBMCs, collected from healthy individuals with different rs6871626 genotypes, were stimulated with LPS to examine the level of IL-10 (a), IL-1β (b), IL-17 (c), IL-8 (d), IFN-γ (e), TNF-α (f), IL-6 (g), and MCP-1 (h) (CC: n = 20–37, AC: n = 28–35, AA: n = 6–7).

FIGURE 2. The effect of rs6871626 on cytokine production. PBMCs, collected from healthy individuals with different rs6871626 genotypes, were stimulated with LPS to examine the level of IL-10 (a), IL-1β (b), IL-17 (c), IL-8 (d), IFN-γ (e), TNF-α (f), IL-6 (g), and MCP-1 (h) (CC: n = 20–37, AC: n = 28–35, AA: n = 6–7).
The current study is the first to show an association of the lncRNAs SNP rs4937362 in RP11-264E20.1 and has been shown to be strongly associated with follicular lymphoma \((P = 6.76 \times 10^{-11})\). To further validate our findings, the other SNPs rs2156698 and rs73013527, which are in strong LD with rs4937362, were also genotyped and the results were consistent with the rs4937362 genotyping data. Intriguingly, rs4937362 locates at approximately 35 kb upstream of ETS1, which plays an important role in autoimmune diseases, such as SLE, psoriasis, RA, MS, pediatric uveitis, and hematopoietic tumors. These findings suggest that RP11-264E20.1 rs4937362 possibility affects disease susceptibility by regulating ETS1. It is not clear why rs4937362 associated with AS’ AAU but not in AS patients with AU. Further studies are needed to investigate whether the manifestations of AS in these two patient groups may differ from one another.

Our study also has several limitations. We only examined the association of IncRNAs SNPs with uveitis or AS in a Chinese Han population, and further studies in other ethnic populations are needed to generalize our findings. This study only included 110 SNPs of IncRNAs identified to be associated with immune-mediated diseases by earlier studies. and it is possible that we may have missed potential not yet discovered IncRNA risk or protective SNPs. In view of the fact that these patients are often on a treatment regimen with immunosuppressive drugs and that it was difficult to collect sufficient patient samples due to the low frequency of the rs6871626 AA genotype (12.4%) and rs4937362 CC genotype (8.4%) in patients, we only examined the expression of cytokines in genotyped healthy controls. Further studies are needed to validate the role of rs6871626 and rs4937362 in uveitis as well as in AS patients.

In conclusion, this study shows novel associations between several IncRNAs SNPs with VKH disease and AS, supporting the role of these neglected gene regions in autoimmune disease.
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