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 3305 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 $P = 9.37 \times 10^{-7}$, 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 $P = 1.88 \times 10^{-4}$, 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] Acute 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 *IL17F*, *IL-23A*, *TNFAIP3*, *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, Th1cells, 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*.[15,16] 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.[17,18] According to the transcript size, ncRNAs can be divided into many types, including microRNAs (miRNAs) and long ncRNAs (lncRNAs).[19] miRNAs range from 19 to 25 nucleotides, and have shown to play important roles in various human immune diseases, including VKH disease, BD, AAU, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), systemic sclerosis, and primary Sjögren's syndrome.[20–32] *miR-146a* had an effect on cytokine production of IL-8, TNF-α, IL-17, and IL-1β in VKH disease.[33] *miR-155* regulated the mRNA expression of *Ets-1* and the Th17 immune response in active BD.[34] Copy number variants of *miR-143*, *miR-146a*, *miR-9-3*, *miR-205*, *miR-301a*, and *miR-23a* were shown to be associated with the susceptibility to AAU.[30] lncRNAs are a group of noncoding potential transcripts that are longer than 200 nucleotides.[19] 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.[17,35,36] A number of studies have suggested that lncRNAs are also involved in the development of autoimmunity.[19,37–43] lncRNAs can regulate the development of various immune cells, such as B and T lymphocytes, macrophages, and dendritic cells (DCs).[44,45] Recent studies showed that several lncRNAs are especially expressed in cells responsible for the innate and adaptive immune response.[44–46] Examples include *LncRNA-CD244*, *lincRNA-Cox2*, *THRIL*, *TH2-LCR*, and *lnc-DC*, which have been shown to be expressed in T cells, macrophages, and DCs.[45] 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.[37,46–50]

A number of studies have shown that the pathogenesis of autoimmune diseases, such as SLE, RA, and MS, were associated with lncRNAs.[19,37–43] *Lnc-USP50–2*, *lnc-ZNF354A-1*, *lnc-FRG2C-3*, and *lnc-LIN54–1* have been shown to be involved in the development of AS.[51]

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.[52] 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 rs6871626 was associated with VKH disease and SNP rs4937362 in *RP11-264E20.1* with the group of AS patients without AAU. 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 2537 controls. The nonparametric Mann-Whitney U test or χ^2 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.[53,54] 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.[55] Optical coherence tomography and ultrasound biomicroscopy were helpful in the diagnosis of AAU.[56] The diagnosis of AS was made according to the 1984 modified criteria of New York.[57] *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@lincTFBS (http://210.46.85.180:8080/SNP_linc_tfbs/, in the public domain), lncRNASNP-human (http://bioinfo.life.hust.edu.cn/lncRNASNP/, in the public domain), and lincSNP (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 for reverse transcription to synthesize cDNA 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′-GCTGCTTCACAAAAAGGAAGATG-3′ and 5′-ACCAGC AGGTGAAACGTCCA-3′; *UBLCP1*: 5′-TCTCGCAGAGTGAAAGA GTACA-3′ and 5′-CTCTGCACAAGACCTGTGGT-3′; *ETS1*: 5′- GCAGCCAGTCATCTTTCAACAGCC-3′ and 5′- TCAGCACGGT CCCGCACATA-3′; *FLI1*: 5′-CCAAAGTGCACGGCAAAAGA-3′ and 5′-GGCATGGTAGGAAGGCATGT-3′; *RP11–264E20.1*: 5′-TGT GACTGTTGAGGGCTGTC-3′ and 5′-CAAGCGCAAAGGGATGT CAC-3′; *LOC285627*: 5′-CTACATTGCTCCCACCACTC-3′ and 5′- TAGTCGTAACATGGGGCTCT-3′; *β-actin*: 5′- GGATGCAGAAG GAGATCACTG-3′ and 5′-CGATCCACACGGAGTACTTG-3′. Relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Culture and ELISA

PBMCs isolated from peripheral blood were seeded at 1×10^6 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 ng/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, Shangahi, 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. $P < 0.05/110 = 4.55 \times 10^{-4}$ 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 lncRNAs SNPs in the First-Phase Study

The frequencies of genotypes and alleles of 110 SNPs of lncRNAs 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 CA genotype, A allele, and a decreased frequency of the CC genotype ($P = 6.04 \times 10^{-5}$, odds ratio [OR] = 1.66; $P = 5.77 \times 10^{-5}$, OR = 1.45; $P = 2.13 \times 10^{-6}$, 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 of the C allele of *RP11-264E20.1*/rs4937362 in the AS without AAU group as compared with controls ($P = 1.28 \times 10^{-4}$, OR = 1.42; $P = 3.66 \times 10^{-5}$, OR = 1.69, $P = 2.11 \times 10^{-5}$, 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 lncRNAs 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, 336 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 ($P = 2.10 \times 10^{-2}$, OR = 0.85, $P = 4.20 \times 10^{-2}$, 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 ($P = 2.00 \times 10^{-3}$, OR = 1.45, $P = 1.00 \times 10^{-3}$, OR = 0.74, respectively) (Table 3). The combined results showed an increased frequency in the

Table 1. Clinical Characteristics of Participants Enrolled in This Study

	First–Stage	%	Second–Stage	%	*P*	Total	%
Patients with BD	384	–	–	–	–	384	–
Mean age ± SD	34.3 ± 8.6	–	–	–	–	34.3 ± 8.6	–
Male	332	86.5	–	–	–	332	86.5
Female	52	13.5	–	–	–	52	13.5
Uveitis	384	100	–	–	–	384	100
Oral ulcer	373	97.1	–	–	–	373	97.1
Genital ulcer	229	59.6	–	–	–	229	59.6
Skin lesions	291	75.8	–	–	–	291	75.8
Arthritis	61	15.9	–	–	–	61	15.9
Positive pathergy test	97	25.3	–	–	–	97	25.3
Patients with VKH	384	–	1242	–	–	1626	–
Mean age ± SD	39.9 ± 14.1	–	39.8 ± 13.8	–	0.89	39.8 ± 13.9	–
Male	207	53.9	704	56.7	0.18	911	56.0
Female	177	46.1	538	43.3	0.18	715	44.0
Uveitis	384	100	1242	100	0.52	1626	100
Headache	175	45.6	529	42.6	0.28	704	43.3
Tinnitus	182	47.4	531	42.7	0.17	713	43.8
Alopecia	150	39.1	430	34.6	0.15	580	35.7
Vitiligo	70	18.2	199	16.0	0.22	269	16.5
Poliosis	152	39.6	424	34.1	0.10	576	35.4
AAU patients	768	–	607	–	–	1375	–
Mean age ± SD	39.5 ± 12.2	–	40.8 ± 11.7	–	–	40.1 ± 12.0	–
Male	481	62.6	345	56.8	–	826	60.1
Female	287	37.4	262	43.2	–	549	39.9
AAU with AS	384	–	240	–	–	624	–
Mean age ± SD	39.2 ± 11.4	–	39.5 ± 11.5	–	0.59	39.4 ± 11.4	–
Male	286	74.5	174	72.5	0.32	460	73.7
Female	98	25.5	66	27.5	0.32	164	26.3
AAU without AS	384	–	367	–	–	751	–
Mean age ± SD	39.8 ± 13.0	–	41.7 ± 11.7	–	0.32	40.7 ± 12.4	–
Male	195	50.8	171	46.6	0.14	366	48.7
Female	189	49.2	196	53.4	0.14	385	51.3
AS without AAU	384	–	336	–	–	720	–
Mean age ± SD	30.7 ± 10.8	–	29.2 ± 9.2	–	0.24	30.0 ± 10.0	–
Male	286	74.5	264	78.6	0.12	550	76.4
Female	98	25.5	72	21.4	0.12	170	23.6
Uveitis	0	0	0	0	–	0	0
HLA-B27(+)*	256	66.7	283	84.2	–	539	74.9
HLA-B27(−)	32	8.3	27	8.0	–	59	8.2
Healthy controls	768	–	2537	–	–	3305	–
Mean age ± SD	39.7 ± 11.0	–	39.9 ± 11.1	–	0.94	39.8 ± 11.1	–
Male	407	53.0	1330	52.4	0.41	1737	52.6
Female	361	47.0	1207	47.6	0.41	1568	47.4

The percentage of *HLA-B27* is the proportion of patients with AS without AAU who were tested for *HLA-B27*.

* As many individuals enrolled in this study did not-test HLA-B27, so the percentage difference of both groups did not perform. The nonparametric Mann–Whitney *U* test or χ^2 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 ($P = 2.29 \times 10^{-6}$, OR = 1.49; $P = 9.37 \times 10^{-7}$, 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 ($P = 1.59 \times 10^{-5}$, OR = 0.76; $P = 1.88 \times 10^{-4}$, 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 ($r^2 = 0.89$) were chosen to confirm the association. The results showed a consistent association of rs2156698 and rs73013527 with AS^+AAU^- ($P = 4.38 \times 10^{-5}$, OR = 0.67; $P = 1.37 \times 10^{-4}$, OR = 0.69, 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*, *FLI1*, 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

SNP	Stage	Genotype/ Allele	VKH		Control		*P*	OR (95%CI)
			n	%	*n*	%		
rs6871626	First (Southwest China)	CC	123	32.9	363	47.7	2.13×10^{-6}	0.54 (0.42–0.70)
		CA	207	55.3	325	42.7	6.04×10^{-5}	1.66 (1.3–2.13)
		AA	44	11.8	73	9.6	0.26	1.26 (0.85–1.87)
		A	295	39.4	471	30.9	5.77×10^{-5}	1.45 (1.21–1.74)
rs6871626	Second (North China, Central China, East China)	CC	507	0.5	1029	44.5	2.10×10^{-2}	0.85 (0.74–0.98)
		CA	588	47.0	1011	43.7	0.06	1.14 (0.99–1.31)
		AA	157	12.5	272	11.7	0.50	1.08 (0.87–1.33)
		A	902	36.0	1555	33.6	4.20×10^{-2}	1.11 (1.00–1.23)
rs6871626	Combined	CC	630	38.7	1392	45.3	1.59×10^{-5}	0.76 (0.68–0.86)
		CA	795	48.9	1336	43.5	3.87×10^{-4}	1.24 (1.10–1.40)
		AA	201	12.4	345	11.2	0.25	1.12 (0.93–1.34)
		A	1197	36.8	2026	33.0	1.88×10^{-4}	1.19 (1.08–1.30)

P value of the first and combined stage less than $0.05/110 = 4.55 \times 10^{-4}$ was considered significant.

Table 3. The Association of rs4937362, rs2156698, and rs73013527 With $AS^{+}AAU^{-}$

SNP	Stage	Genotype/ Allele	$AS^{+}AAU^{-}$		Control		*P*	OR (95%CI)
			n	%	*n*	%		
rs4937362	First (Southwest China)	CC	32	8.5	104	13.6	1.20×10^{-2}	0.59 (0.39–0.89)
		CT	152	40.2	366	47.9	1.40×10^{-2}	0.73 (0.57–0.94)
		TT	194	51.3	294	38.5	3.66×10^{-5}	1.69 (1.31–2.16)
		C	216	28.6	574	37.6	2.11×10^{-5}	0.67 (0.55–0.80)
rs4937362	Second (North China, Central China, East China)	CC	28	8.4	242	11.9	0.06	0.68 (0.45–1.02)
		CT	130	38.9	911	44.6	0.05	0.79 (0.62–1.00)
		TT	176	52.7	887	43.5	2.00×10^{-3}	1.45 (1.15–1.83)
		C	186	27.8	1395	34.2	1.00×10^{-3}	0.74 (0.62–0.89)
rs4937362	Combined	CC	60	8.4	346	12.3	4.00×10^{-3}	0.65 (0.49–0.87)
		CT	282	39.6	1277	45.5	4.00×10^{-3}	0.78 (0.66–0.93)
		TT	370	52	1181	42.2	2.29×10^{-6}	1.49 (1.26–1.75)
		C	402	29	1969	35.1	9.37×10^{-7}	0.73 (0.64–0.83)
rs2156698*		GG	219	57	337	44.4	5.45×10^{-5}	1.66 (1.30–2.13)
		GA	138	36	338	44.5	5.00×10^{-3}	0.70 (0.54–0.90)
		AA	27	7	84	11.1	3.00×10^{-2}	0.61 (0.39–0.96)
		A	192	25	506	33.3	4.38×10^{-5}	0.67 (0.55–0.81)
rs73013527*		CC	206	53.6	311	41.6	1.23×10^{-4}	1.62 (1.27–2.08)
		CT	147	38.3	347	46.5	9.00×10^{-3}	0.72 (0.56–0.92)
		TT	31	8.1	89	11.9	4.70×10^{-2}	0.65 (0.42–1.00)
		T	209	27.2	525	35.1	1.37×10^{-4}	0.69 (0.57–0.84)

P value of the first and combined stage less than $0.05/110 = 4.55 \times 10^{-4}$ was considered significant.
* SNPs rs2156698 and rs73013527 linked with rs4937362 (r2 = 0.89) were chosen to confirm the association between rs4937362 and AS.

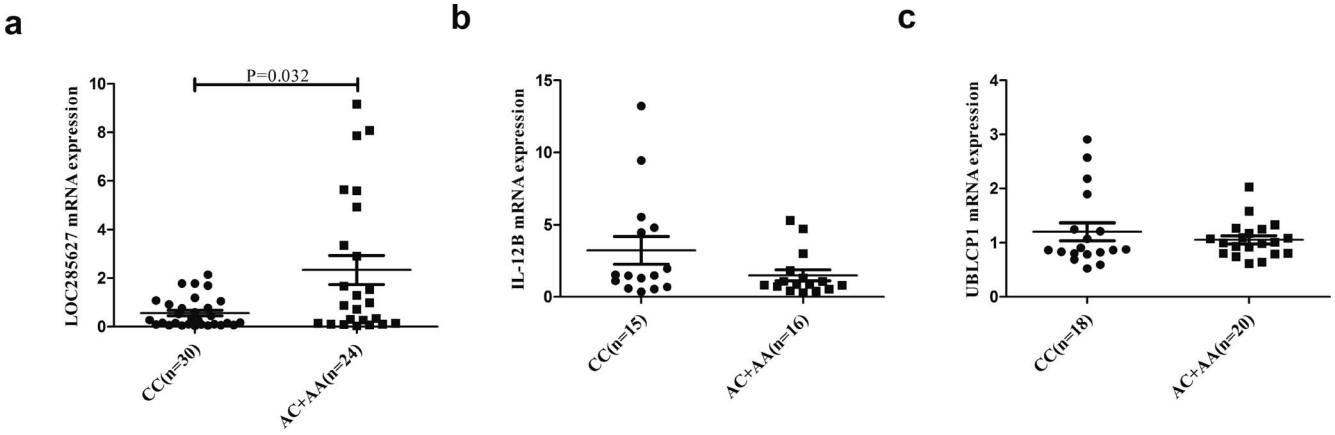

Figure 1. The influence of diverse genotypes of rs6871626 on the expression of *LOC285627* (CC: $n = 30$, AC+AA: $n = 24$), *IL12B* (CC: $n = 15$, AC+AA: $n = 16$), *UBLCP1* (CC: $n = 18$, AC+AA: $n = 20$), in PBMCs of healthy individuals. The mRNA expression levels of *LOC285627* (**a**), *IL12B* (**b**), and *UBLCP1* (**c**) were examined in PBMCs of healthy individuals with a variety of genotypes of rs6871626.

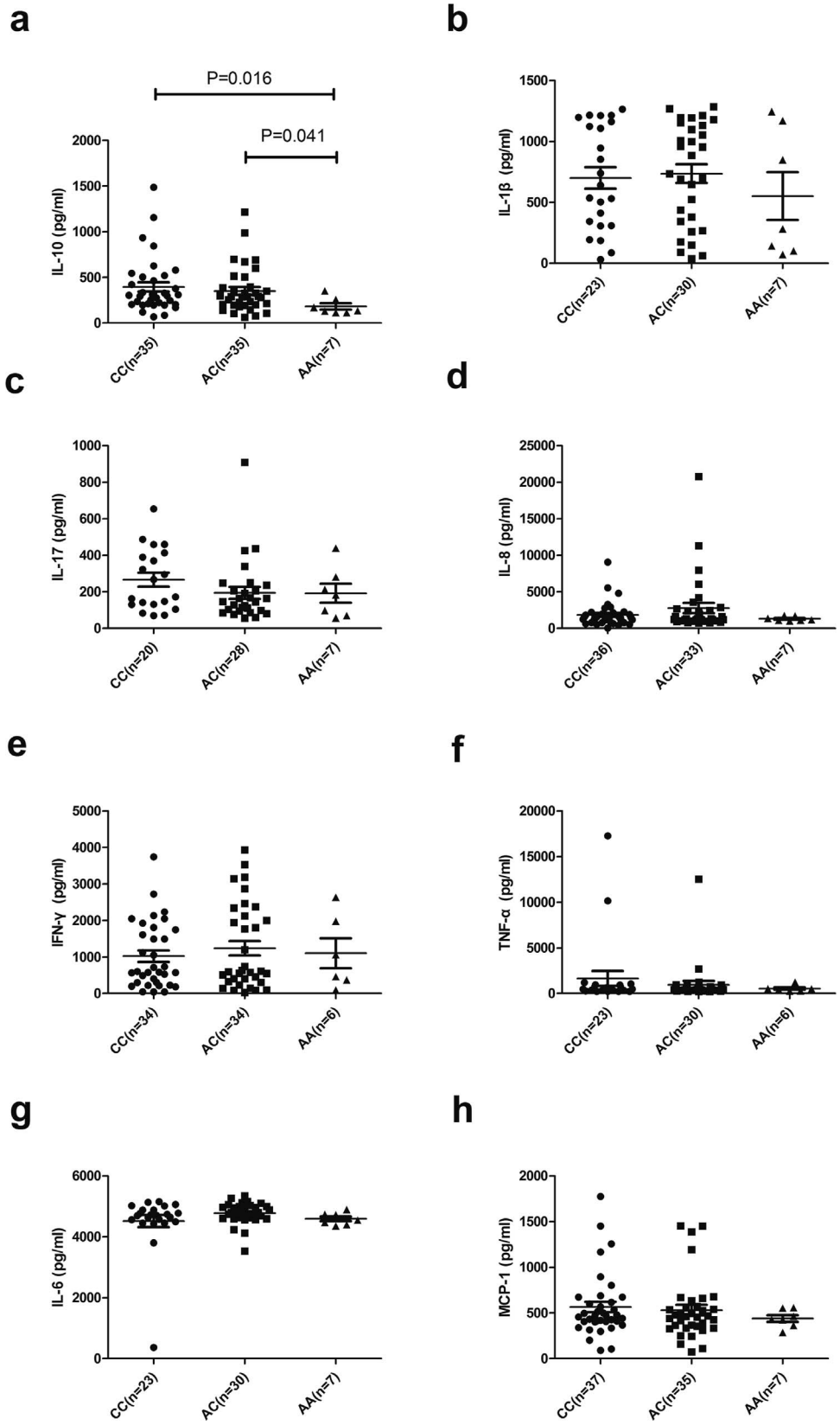

FIGURE 2. The effect of rs6871626 on cytokine production. PBMCs, collected from healthy individuals with different rs6871626 genotypes, were stimulated with LPS to examine the level of IL-10 (**a**), IL-1β (**b**), IL-17 (**c**), IL-8 (**d**), IFN-γ (**e**), TNF-α (**f**), IL-6 (**g**), and MCP-1 (**h**) (CC: $n = 20–37$, AC: $n = 28–35$, AA: $n = 6–7$).

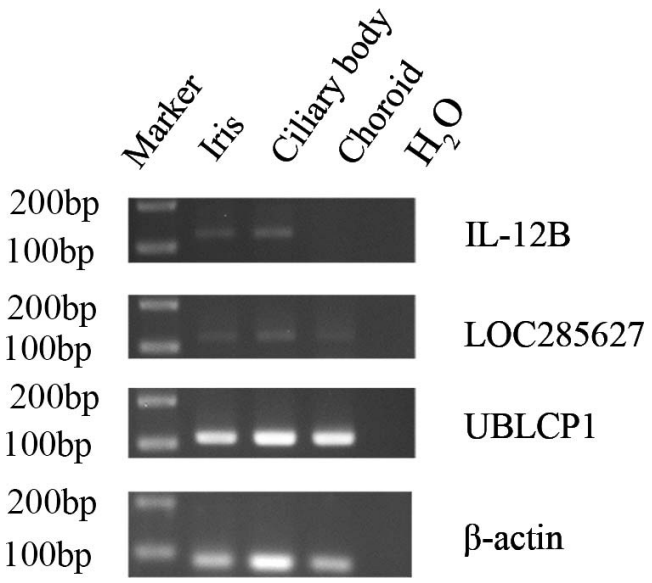

FIGURE 3. The mRNA expression of *IL12B*, *LOC285627*, *UBLCP1*, and *β-actin* in human uveal tissues (iris, ciliary body, and choroid) from a 19-year-old male who died from leucocythemia.

that individuals with the rs6871626/CC genotype had a decreased *LOC285627* mRNA expression as compared with AC/AA individuals (Fig. 1a, $P = 0.032$). However, we did not find any differences between *IL12B*, *UBLCP1*, *RP11-264E20.1*, *ETS1*, *FLI1* mRNA expression and the various genotypes of rs6871626 or rs4937362 (Figs. 1b, 1c; Supplementary Figs. S2a–c).

The Influence of rs6871626 and rs4937362 Genotypes on Cytokine Production

The secretion of cytokines was examined in supernatants of PBMC cultures from healthy individuals following a 24-hour in vitro stimulation with LPS. The concentrations of IL-10, IL-1β, IL-17, IL-8, IFN-γ, TNF-α, IL-6, and MCP-1 were detected using ELISA (Fig. 2, Supplementary Fig. S3). An increased IL-10 production was found in CC carriers as compared with AA carriers ($P = 0.016$) (Fig. 2a). No association of the other cytokines with rs6871626 and rs4937362 genotypes was found (Figs. 2b–h, Supplementary Fig. S3).

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

The expression of *IL12B*, *LOC285627*, and *UBLCP1* was examined in human uveal tissues. The results showed that all three genes were expressed in human iris and ciliary body. Moreover, *LOC285627* and *UBLCP1* were found also to be expressed in the human choroid (Fig. 3).

DISCUSSION

The current study is the first to show an association of the lncRNAs SNP rs4937362 in *RP11-264E20.1* in patients with AS who are not affected with uveitis. In uveitis patients we found that the SNP rs6871626 near the *LOC285627*, *IL12B*, and *UBLCP1* genes is associated with VKH disease. *LOC285627*, *IL12B*, and *UBLCP1* were expressed in human uveal tissues. Our study adds to the existing knowledge that has revealed that lncRNAs play important roles in the development of autoimmunity.[37–43,51]

Functional studies showed a decreased mRNA expression of *LOC285627* in PBMCs in carriers of the CC genotype (VKH protective genotype) of rs6871626 as compared with the carriers of other genotypes and a significantly increased IL-10 production by LPS-stimulated PBMCs in rs6871626 carriers of the CC genotype as compared with carriers of the AA genotype. The increased production of an anti-inflammatory cytokine such as IL-10 in CC genotype carriers might provide an explanation for its protective role in disease susceptibility.

SNP rs6871626 is located between *UBLCP1*, *IL12B*, and *LOC285627* at 5q33.3.[58] Several studies showed that rs6871626 was associated with leprosy, Crohn's disease, Takayasu arteritis, and ulcerative colitis.[58–60] The current study showed that rs6871626 was associated with VKH disease but not with AS. Our study is in agreement with a previous study that showed that rs6871626 was not associated with the susceptibility to AS in mainland Han Chinese.[61] The reason why rs6871626 is only associated with VKH disease but not with the other uveitis groups tested (AAU or BD) is not clear, but may be due to the different etiology between these uveitis entities. BD is an inflammatory disorder with a complex genetic background characterized by uveitis, oral aphthae, skin lesions, and genital ulcers, whereas VKH disease is a systemic autoimmune disease that targets melanocytes not only in the uvea, but also elsewhere in the body, resulting in multiple systemic abnormalities leading to dysacusis, alopecia, poliosis, and vitiligo.[62,63]

As mentioned above, rs4937362 was associated with AS in our Chinese Han population but not with the uveitis entities tested. The SNP rs4937362 is located at 11q24.3 in lncRNA *RP11-264E20.1* and has been shown to be strongly associated with follicular lymphoma ($P = 6.76 \times 10^{-11}$).[64] 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.[65,66] 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 AAU. 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 lncRNAs 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 lncRNAs identified to be associated with immune-mediated diseases by earlier studies. and it is possible that we may have missed potential not yet discovered lncRNA 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 lncRNAs SNPs with VKH disease and AS, supporting the role of these neglected gene regions in autoimmune disease.

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