Association Between Copy Number Variations of *TLR7* and Ocular Behçet's Disease in a Chinese Han Population

Jing Fang,[1,2] Lu Chen,[1] Jihong Tang,[1] Shengping Hou,[1] Dan Liao,[1] Zi Ye,[1] Chaokui Wang,[1] Qinfeng Cao,[1] Aize Kijlstra,[3] and Peizeng Yang[1]

[1]The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology and Chongqing Eye Institute, Chongqing, China
[2]Department of Ophthalmology, Children's Hospital, Chongqing Medical University, Chongqing, China
[3]University Eye Clinic Maastricht, Maastricht, Limburg, The Netherlands

Correspondence: Peizeng Yang, The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology and Chongqing Eye Institute, Chongqing, China, 400016; peizengycmu@126.com.

JF, LC, and JT contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. The purpose of this study was to test whether gene copy number variations (CNVs) of Toll-like receptors (TLRs) are associated with uveitis.

Methods. Copy number variations of TLRs were detected by real-time PCR. The first stage of the study consisted of enrolling 400 Behçet's disease (BD) patients, 400 Vogt-Koyanagi-Harada syndrome patients, 400 patients with acute anterior uveitis associated with or without ankylosing spondylitis, and 600 healthy subjects. The second stage included another set of 578 BD patients and 1000 healthy controls. The frequencies of TLR gene copy number types (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR7*, *TLR9*, *TLR10*) were compared among patients and controls by using the χ^2 test. Real-time PCR was used to detect mRNA expression from peripheral blood mononuclear cells (PBMCs) obtained from healthy controls following stimulation with the *TLR7* agonist R848. Levels of TNF-α, IL-6, IL-1β, and IFN-β in culture supernatants were measured by ELISA.

Results. All TLRs tested, except for *TLR7*, had a gene copy number of two in more than 98% of individuals tested. In the first stage, we found a significantly increased frequency of more than one copy of *TLR7* (located on the X chromosome) in male BD patients and more than two copies in female patients (correction of *P* value [P_C] = 0.021; $P_C = 0.048$, respectively). A second stage and combined study confirmed the association ($P_C = 1.14 \times 10^{-6}$; $P_C = 9.12 \times 10^{-5}$, respectively). *TLR7* mRNA expression in PBMCs was increased in healthy male carriers having more than one copy of *TLR7* or females having more than two copies following stimulation with R848 ($P = 0.021$, $P = 0.006$, respectively). No effect of the various *TLR7* copies on the release of TNF-α, IL-6, IL-1β, and IFN-β could be detected.

Conclusions. This study provides evidence that a high copy number of *TLR7* confers risk for BD in a Chinese Han population. (http://www.chictr.org number, ChiCTR-CCC-12002184.)

Keywords: acute anterior uveitis, ankylosing spondylitis, Behçet's disease, copy number variants, Toll-like receptor 7, Toll-like receptor, uveitis, Vogt-Koyanagi-Harada syndrome

Uveitis, which can manifest as an isolated ocular disease, is an intraocular inflammation. It is regarded as one of the main global causes of blindness and can be caused by infectious or noninfectious mechanisms.[1,2] Uveitis can also be a manifestation of a systemic autoimmune or autoinflammatory disease, which includes Behçet's disease (BD) or Vogt-Koyanagi-Harada (VKH) syndrome.[3] Both the pathogenesis and the clinical manifestations of these disorders show marked differences, however. BD, for instance, is characterized by nongranulomatous uveitis, recurrent oral ulceration, genital ulcers, and skin lesions and is considered a chronic systemic autoinflammatory disorder.[4] VKH syndrome is a multisystem disorder that is presumably caused by an autoimmune response against pigmented tissues leading to inflammatory reactions in the eye, skin, inner ear, and meninges.[5–7] Previous studies have shown that an aberrant innate or adaptive immune response can lead to uveitis syndromes mentioned above, and these uveitis syndromes have a genetic background.[8]

Copy number variants (CNVs), characterized by insertions, deletions, and duplications of genomic sequences ranging from a kilobase to multiple megabase-long pairs, are one of the major contributors to human genetic diversity.[9–12] It is becoming clear that gene copy number can influence the expression of genes.[13,14] Recently, several studies have shown that CNVs of genes involved in the immune response are involved in the pathogenesis of autoimmune disease.[13,15]

Toll-like receptors (TLRs), thought to be one of the links between infection and autoinflammatory or autoimmune disease, are members of the family of pattern recognition receptors (PRRs) together with RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). Thirteen distinct mammalian TLRs have been identified to date, with 10 functional TLRs in humans (*TLR1* to –10).[16] TLRs have been reported to be involved in the pathogenesis of many inflammatory diseases including uveitis.[17,18] Several TLRs including *TLR2*, *TLR3*, *TLR4*, *TLR7*, *TLR8*, and *TLR9* were reported to be associated with BD, VKH syndrome, and anterior uveitis with regard to immunologic

mechanisms involved but also in relation to an immunogenetic background, as shown by the association of these diseases with TLR single nucleotide polymorphisms (SNPs).[19–22] Identifying disease associations with certain TLR gene CNVs is one of the approaches to studying the role of TLRs. Evidence emerging from recent publications indicates that several diseases including autoimmune diseases are associated with CNVs of certain TLR genes or genes related to TLRs.[23–26]

Because TLRs play such an important role in uveitis and because the role of TLR gene copy number has not yet been reported for clinical uveitis, we decided to expand our earlier TLR studies[19,21,22] and questioned whether the CNVs of TLRs might also play a role in the pathogenesis of uveitis. We chose three uveitis entities, BD, VKH syndrome, and acute anterior uveitis (AAU) associated with ankylosing spondylitis (AS) or not (AAU ± AS), because these diseases are known to belong to the category of autoinflammatory or autoimmune diseases in whose pathogenesis TLRs are known to play a role and because these entities are relatively common in China and, therefore, allow the collection of large sample numbers, providing sufficient statistical power to reach a meaningful conclusion.

Materials and Methods

Patients and Controls Study Population

This was a prospective case-control study. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China (permit number: 2009-201008). The tenets of the Declaration of Helsinki were followed in all procedures. Written informed consent was given by all participants in this study. This study was registered with the Chinese Clinical Trial Registry, number ChiCTR-CCC-12002184.

The study was designed as two-stage prospective case-control association research. A total of 400 ocular BD patients, 400 VKH syndrome patients, 400 AAU ± AS patients, and 600 normal controls, who were recruited from a Chinese Han populations (the individuals of the groups mentioned above were referred to the First Affiliated Hospital of Chongqing Medical University, Chongqing or the Zhongshan Ophthalmic Center, Sun Yat-sen University, China), were enrolled in the first stage of the study. Stage two consisted of an additional set of 578 ocular BD patients and 1000 normal Chinese Han subjects. All individuals in this study came from the First Affiliated Hospital of Chongqing Medical University, Chongqing, or the Zhongshan Ophthalmic Center, Sun Yat-sen University, China. The two stages were analyzed independently. All controls were enrolled between April 2005 and February 2014. The clinical features of the ocular BD patients who fulfilled the criteria of the International Study Group,[27] are presented in the Results. Clinical characteristics of the patients with VKH syndrome, which was diagnosed based on First International Workshop criteria for VKH syndrome, were recorded during the whole course of follow-up and are summarized in the Results. Patients had AAU diagnosed principally according to clinical manifestations,[28,29] and patients with AS fulfilled modified New York criteria. Clinical characteristics of the AAU patients with or without AS are also presented in the Results.

Analysis of TLR Gene Copy Number Variations and Genomic DNA Extraction

Extraction of peripheral blood genomic DNA was done with QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), as described previously.[13] As no CNVs of *TLR4* and *TLR8* were found in the database of genomic variants (www.tcag.ca/dgv/app/home, in the public domain), the two TLRs were excluded from our study. Toll-like receptor gene CNVs were performed in 96-well plates with a model 7500 real-time PCR system, following the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). *TLR1* (catalog no. Hs00395312_cn), *TLR2* (catalog no. Hs00871640_cn), *TLR3* (catalog no. Hs01233552_cn), *TLR5* (catalog no. Hs01059073_cn), *TLR6* (catalog no. Hs02685381_cn), *TLR7* (catalog no. Hs00226289_cn), *TLR9* (catalog no. Hs00787794_cn), and *TLR10* (catalog no. Hs02871353_cn) were detected using TaqMan assays labeled with 6-carboxy-fluorescein (FAM; Applied Biosystems), respectively.

Using the maximum likelihood method, we analyzed absolute quantitation raw data (using ΔCt values) with CopyCaller software v2.0 (Applied Biosystems). We assumed 2 was the most frequent number of gene copies for the TLR genes (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR9*, and *TLR10*) and for the reference, respectively. TaqMan RNase P assay (TaqMan Copy Number Reference Assay; Applied Biosystems) labeled with green fluorescent dye (VIC; Applied Biosystems) was used as an internal copy number reference. Because the *TLR7* gene is located on chromosome X, we assumed 2 was the most frequent number of gene copies for the *TLR7* gene in female patients and female controls, and we assumed 1 in male patients and male controls, as described previously.[25] Every experiment was repeated three times. If a sample detected showed undetectable reference signal (VIC Ct > 32), zero copy number, or a confidence interval in the copy number prediction lower than 0.95, the sample was omitted from further analysis.

mRNA Expression of the *TLR7* Gene

Peripheral blood mononuclear cells (PBMCs), which were obtained from healthy individuals, prepared from heparinized blood by Ficoll-Hypaque density gradient centrifugation (GE Medical, Piscataway, NJ, USA). Extraction of total RNA was done from PBMCs with or without stimulation of the *TLR7* agonist R848 (2.5 μg/mL; Alexis Biochemicals, San Diego, CA, USA) for 72 hours at a density of 1×10^6 cells/mL, using TRIzol (Invitrogen, Grand Island, NY, USA), followed by reverse transcription using a transcriptase kit (Takara Biosystems, Tokyo, Japan). Real-time quantitative PCR was performed to compare mRNA expression of the *TLR7* gene (sense primer: 5′ TTAACCTGGATGGAAACCAGCTA 3′; antisense primer: 5′ TCAAGGCTGAGAAGCTGTAAGCTA 3′), using the 7500 system with the SYBR Green I assay kit (Applied Biosystems). Data were normalized to mRNA of β-actin as reported in previous studies.[22,30,31] Assays were performed using model 7500 real-time PCR system (Applied Biosystems). Relative expression levels were calculated using the 2-ΔΔCt method.

Flow Cytometric Analysis

Flow cytometric intracellular staining was used for *TLR7*. Briefly, PBMCs were stimulated with *TLR7* agonist R848 and then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen, San Diego, CA, USA). Next, cells were incubated with *TLR7* antibody or isotype control antibody (mouse immunoglobulin G [IgG] 2A, clone 20102). Fluorescent antibodies (human *TLR7*-phycoerythrin–conjugated antibody) were obtained from R&D Systems (Minneapolis, MN, USA). Samples were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest software (BD Biosciences). Results were expressed as percentage differences compared with isotypic control (IC)

Table 1. Clinical Features of Ocular Behçet's Disease Study Patients

Clinical Features	Patients with BD	
	n, Total = 978	% of Total
Uveitis	978	100
Oral ulcer	978	100
Genital ulcer	504	51.5
Skin lesions	648	66.3
Pathergy reaction	258	26.4
Hypopyon	214	21.9
Arthritis	159	16.3

using the following formula: ([MFI of TLR − MFI of IC]/MFI of IC × 100%) (where MFI is mean fluorescence intensity).

Cytokine Measurements

Peripheral blood mononuclear cells obtained from venous blood of healthy individuals were stimulated with *TLR7* agonist R848 (2.5 μg/mL) for 72 hours at a density of 1×10^6 cells/mL. The concentrations of TNF-α, IL-6, IL-1β, and IFN-β in cell culture supernatants were detected using an ELISA Development kit (R&D Systems).

Statistical Analysis

Analysis of real-time PCR data was done using model 7500 version 2.0.6 software (Applied Biosystems). Examination of relative gene copy numbers was performed with the comparative Ct method, using CopyCaller version 2.0 software (Applied Biosystems). Differences between the frequencies of TLR CNVs in patients and those in healthy individuals were analyzed with the χ^2 test, using SPSS version 17.0 software (IBM, Armonk, NY, USA). We corrected the *P* values (P_C) with the Bonferroni correction by multiplying with the number of analyses performed. A number of 21 independent comparisons was used as the basis of the Bonferroni corrections in this study.

Results

Clinical Characteristics of the Enrolled Patient Groups

Distributions of clinical characteristics of the BD, VKH syndrome, and AAU ± AS patients were collected at the time of diagnosis and presented in Table 1 and in Supplementary Tables S1 and S2, respectively.

Association of the TLR Gene Copy Number Variants in Patients With Behçet's Disease

The copy number of all TLRs tested, except for *TLR7*, was 2 in more than 98% of patients and healthy controls. Significant variations were observed for the gene copy numbers of *TLR7* in both BD patients and controls. Because the *TLR7* gene is located on chromosome X, its copy number is affected by sex. The CNV analysis for *TLR7* was performed in a total of 400 BD patients (male-to-female ratio of 311:89) and 600 healthy controls (male-to-female ratio of 325:275). We found significantly increased frequencies of male BD patients who had more than one copy of *TLR7* and female patients who had more than two copies ($P_C = 0.021$; odds ratio [OR] = 2.452; 95% confidence interval [CI] = 1.440–4.176; $P_C = 0.048$; OR = 2.620, 95% CI = 1.406–4.882, respectively) (Table 2). A second stage confirmatory study including an additional and separate group of 578 BD patients (male-to-female ratio of 486:92) and 600 health controls (male-to-female ratio of 688:312) was subsequently carried out. Copy number variation analysis of the *TLR7* gene of the second stage and combined first and second stages of study was also carried out separately, according to sex. In the stage two and combined studies, we confirmed the association of the increased frequency of having more than one copy of *TLR7* in males and having more than two copies in females with ocular BD ($P_C = 1.14 \times 10^{-6}$; OR = 2.324, 95% CI = 1.680–3.215; $P_C = 9.12 \times 10^{-5}$; OR = 2.492, 95% CI = 1.603–3.873, respectively) (Table 2).

Table 2. Copy Number Variants of the *TLR7* Gene in Ocular Behçet's Disease

Gene	Stage	Copy Number	Case *n* (Frequency)	Control *n* (Frequency)	*P* Value	P_C Value	OR (95% CI)
TLR7 (male)	First	<1	4 (0.013)	9 (0.028)	0.186	NS	0.457 (0.139–1.501)
		1	260 (0.836)	294 (0.905)	0.01	NS	0.538 (0.334–0.866)
		>1	47 (0.151)	22 (0.068)	0.001	0.021	2.452 (1.440–4.176)
	Replication	<1	10 (0.021)	15 (0.022)	0.886	NS	0.943 (0.420–2.116)
		1	415 (0.854)	631 (0.917)	0.001	0.006	0.528 (0.365–0.764)
		>1	61 (0.126)	42 (0.062)	1.20×10^{-4}	7.20×10^{-4}	2.208 (1.463–3.332)
	Combined	<1	14 (0.018)	24 (0.024)	0.367	NS	0.737 (0.379–1.434)
		1	675 (0.847)	925 (0.913)	1.27×10^{-5}	7.62×10^{-5}	0.526 (0.393–0.705)
		>1	108 (0.136)	64 (0.063)	1.90×10^{-7}	1.14×10^{-6}	2.324 (1.680–3.215)
TLR7 (female)	First	<2	16 (0.180)	50 (0.182)	0.965	NS	0.986 (0.530–1.837)
		2	52 (0.584)	196 (0.711)	0.024	NS	0.566 (0.345–0.930)
		>2	21 (0.236)	29 (0.107)	0.002	0.042	2.620 (1.406–4.882)
	Replication	<2	19 (0.207)	60 (0.192)	0.763	NS	1.093 (0.613–1.948)
		2	53 (0.576)	221 (0.708)	0.017	NS	0.560 (0.346–0.904)
		>2	20 (0.217)	31 (0.101)	0.003	0.018	2.518 (1.356–4.675)
	Combined	<2	35 (0.193)	110 (0.187)	0.857	NS	1.040 (0.681–1.587)
		2	105 (0.580)	417 (0.710)	0.001	0.006	0.563 (0.399–0.795)
		>2	41 (0.227)	60 (0.102)	1.52×10^{-5}	9.12×10^{-5}	2.492 (1.603–3.873)

CI, confidence interval; NS, nonsignificant; OR, odds ratio.

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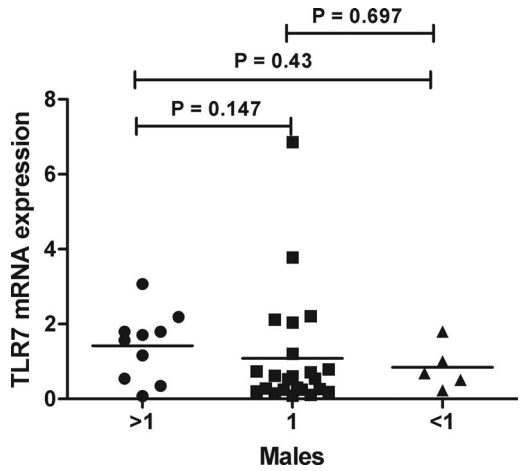

FIGURE 1. mRNA expression of different copies of the *TLR7* gene in males by nonstimulated PBMCs. *TLR7* expression was not significantly different between different copy numbers.

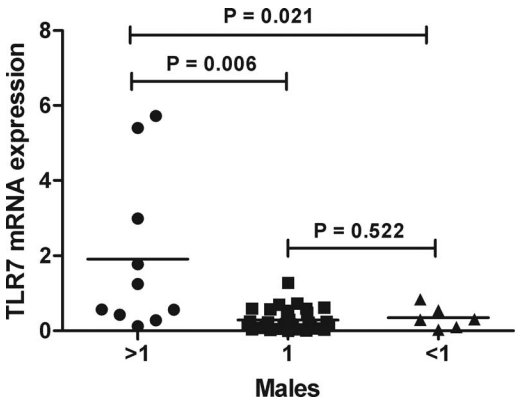

FIGURE 3. mRNA expression of different copies of the *TLR7* gene in males by *TLR7* agonist R848-stimulated PBMCs. *TLR7* expression in carriers having more than one copy was significantly higher than in individuals carrying one copy or less than one copy ($P = 0.006$, $P = 0.021$, respectively).

Association of the TLR Gene Copy Number Variants in Patients With VKH Syndrome or AAU Associated With AS or Not

As mentioned above, the CNV analysis of all TLRs tested show a variation only for *TLR7*. Analysis of *TLR7* CNV was also performed in VKH syndrome and AAU ± AS patients and controls. A total of 400 VKH patients (male-to-female ratio of 198:202), 400 AAU ± AS patients (male-to-female ratio of 205:195), and 600 healthy controls (male-to-female ratio of 325:275) were included, and a separate sex analysis was also done. We failed to find an association between *TLR7* gene copy numbers with VKH syndrome or AAU ± AS (Supplementary Tables S3 and S4, respectively).

Relationship Between Copy Numbers of *TLR7* and the Corresponding Gene Expression at the mRNA Level

Because we found that CNVs of *TLR7* showed an association with susceptibility to ocular BD, we investigated whether a different copy number might affect the expression of *TLR7* under normal or inflammatory conditions. mRNA expression of *TLR7* was detected in PBMCs obtained from healthy controls, by real-time PCR. We analyzed healthy individuals for the *TLR7* CNVs and then investigated *TLR7* mRNA expression by PBMCs with or without stimulation with the *TLR7* agonist R848, using real-time PCR. No differences in gene expression could be found between the presence of various copies of the *TLR7* gene in males or females when PBMCs were not stimulated (Figs. 1, 2). Following stimulation with the *TLR7* agonist R848, carriers with more than one copy in normal male subjects had a higher expression of *TLR7* mRNA than normal subjects carrying one copy or more than one copy ($P = 0.006$; $P = 0.021$, respectively) (Fig. 3), and carriers with more than two copies in female individuals had higher *TLR7* mRNA expression than normal subjects carrying two copies or less than two copies ($P = 0.006$; $P = 0.003$, respectively) (Fig. 4). No significant differences were observed between carriers of one copy and those with less than one copy of *TLR7* in male individuals or between those carrying two copies and those carrying less than two copies of *TLR7*, but this could be due to the fact that the numbers of individuals carrying less than one copy of *TLR7* in males and less than two copies of *TLR7* in females were very low ($n = 6$; $n = 5$, respectively).

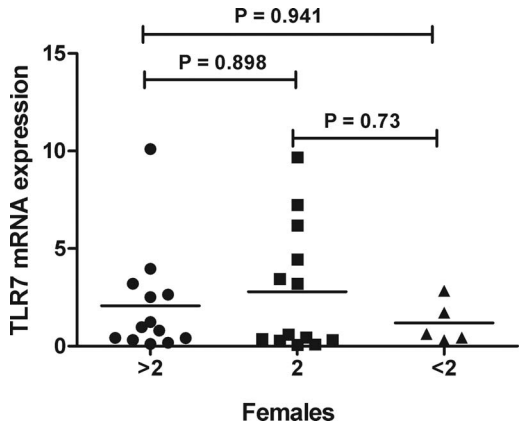

FIGURE 2. mRNA expression of different copies of the *TLR7* gene in females by nonstimulated PBMCs. *TLR7* expression was not significantly different between different copy numbers.

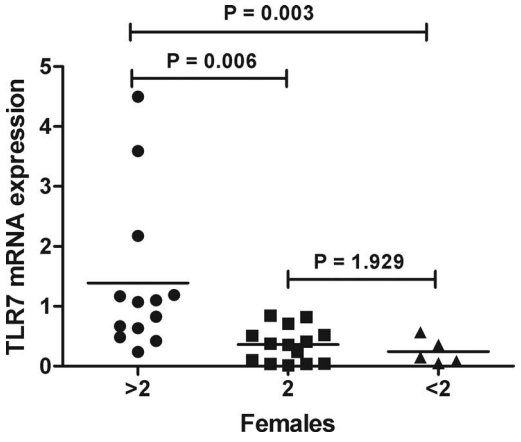

FIGURE 4. mRNA expression of different copies of the *TLR7* gene in females by *TLR7* agonist R848-stimulated PBMCs. *TLR7* expression in carriers having more than two copies was significantly higher than in individuals carrying two copies or less than two copies ($P = 0.006$, $P = 0.003$, respectively).

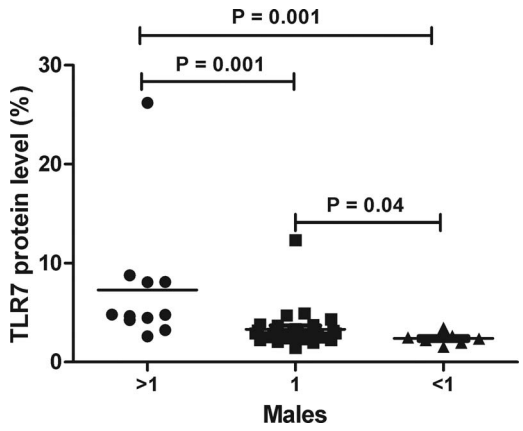

FIGURE 5. Protein levels of *TLR7* in PBMCs of male carriers having a different gene copy number following stimulation with the *TLR7* agonist R848. Protein level of *TLR7* in male carriers having more than one copy was significantly higher than in individuals carrying one copy or less than one copy ($P = 0.001$, $P = 0.001$, respectively).

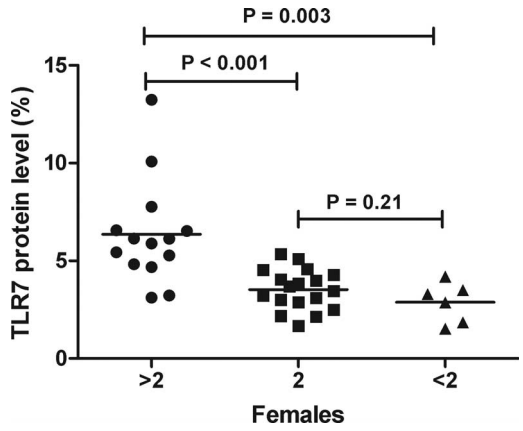

FIGURE 6. Protein levels of *TLR7* in PBMCs of female carriers having a different gene copy number following stimulation with the *TLR7* agonist R848. Protein level of *TLR7* in female carriers having more than two copies was significantly higher than in individuals carrying two copies or less than two copies ($P < 0.001$, $P = 0.003$, respectively).

Relationship Between Copy Numbers of *TLR7* and Corresponding Protein Levels

Furthermore, we measured the protein levels in PBMCs following stimulation with the *TLR7* agonist R848 in male and female carriers who had a known copy number. We found that carriers with more than one copy in male normal individuals had a higher *TLR7* protein level than normal subjects carrying one copy or less than one copy ($P = 0.001$; $P = 0.001$, respectively) (Fig. 5), and carriers with more than two copies in female individuals had a higher protein level of *TLR7* than normal subjects carrying two copies or less than two copies ($P < 0.001$; $P = 0.003$, respectively) (Fig. 6).

Relationship Between Different Copy Numbers of *TLR7* and Downstream Inflammatory Factors

The aforementioned result showed that different copies of *TLR7* in male or female individuals could affect *TLR7* gene expression. In a next series of experiments, we tested whether different copies of *TLR7* in male or female normal individuals could also affect the expression of downstream cytokines secreted by PBMCs following stimulation with the *TLR7* agonist R848. The production of TNF-α, IL-6, IL-1β, and IFN-β, which are important *TLR7* downstream factors[32,33] in PBMC culture supernatants by ELISA, was further measured. However, we found no effect of the various *TLR7* copies in males or females on the release of these three cytokines (data not shown).

DISCUSSION

In this study, we analyzed CNVs of several TLRs (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR7*, *TLR9*, and *TLR10*) in BD, VKH syndrome, and AAU ± AS patients and compared the frequency with those in healthy controls. The results showed that having more than one copy of the *TLR7* gene in males or two copies in females significantly increased the risk of developing BD. However, no significant differences were found between different copy numbers of *TLR7* and those of VKH syndrome or AAU ± AS patients. No association was found between gene copy number and uveitis for the other seven TLRs (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR9*, *TLR10*) investigated, but this is not surprising because more than 98% of individuals have two copies of these TLRs. To our knowledge, our study is the first to address the role of TLR gene copy numbers in patients with uveitis. We only tested the role of *TLR7* CNV in a number of common uveitis entities seen in China, and further studies are needed to investigate whether an association can also be found in other types of intraocular inflammation that were not covered by our study.

Behçet's disease is one of the most commonly seen uveitis entities in China,[4] and evidence is mounting to show that both environmental as well as genetic factors play an important role in the development of this disease.[8,34,35] *TLR7* is expressed by human plasmacytoid dendritic cells and specifically recognizes single-stranded RNA derived from viruses or immune complexes associated with self-RNA and is one of two X-linked TLR genes.[36] It has been postulated that CNVs in *TLR7* are related to the modulation of the autoimmune response to nuclear material.[37] Previous studies showed that CNVs of *TLR7* had an association with childhood onset systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Graves' disease.[24–26] However, no variation was found in the relative copy number of the *TLR7* gene in adult patients with SLE compared to controls.[38]

We also did not find an association between *TLR7* gene CNV and VKH syndrome or AAU ± AS. The fact that the unique pathogenetic mechanisms for BD are different from these other two ocular inflammatory diseases may lead to the discrepancy between the other uveitis cohorts studied and ocular BD. Abundant evidence is now available to show that BD is an autoinflammatory disease caused by an aberrant response against infectious agents, which might also explain the role of TLRs in its pathogenesis.[39]

Because the CNVs of *TLR7* were shown to have an association with ocular BD and because *TLR7* is located on the X chromosome, we tested whether the expression of *TLR7* could be affected by the different copy numbers in either female or male individuals. Data obtained from our study showed that when PBMCs from healthy male individuals were stimulated with the *TLR7* agonist R848, there was an increased expression of *TLR7* mRNA in the male individuals having more than one copy of the *TLR7* gene compared with those carrying one copy or less than one copy. Interestingly, an increased expression of *TLR7* mRNA in female individuals having more than two copies of *TLR7* gene compared with those carrying two copies or less than two copies was also found when PBMCs from healthy female individuals were stimulated with R848. In addition to mRNA expression, CNV of *TLR7* also had

an effect on its protein expression. We found that carriers with more than one copy in male normal individuals had a higher level of *TLR7* protein than normal subjects carrying one copy or less than one copy, and carriers with more than two copies in female individuals had a higher level of *TLR7* protein than normal subjects carrying two copies or less than two copies. We were not able to show an effect of *TLR7* gene copy number on the expression of certain downstream cytokines such as TNF-α, IL-6, IL-1β, and IFN-β. Earlier studies from Japan did not find an association between *TLR7* SNPs and BD, which suggests that gene variants other than the CNVs for *TLR7* described by us may not be involved in the pathogenesis of this disease.[40]

In addition to *TLR7*, it was reported that other TLRs (*TLR2*, *TLR3*, *TLR4*, *TLR8*, *TLR9*) were involved in several autoimmune diseases.[19,22,41] Only a few studies have addressed the relationship between the copy number of these TLRs and autoimmune disease. No variation was reported in the copy number of the *TLR2* or *TLR4* gene in patients with celiac disease and normal controls.[42] Similar to this result, we found no CNVs of *TLR2* and *TLR3* in the Chinese Han population studied. We also investigated the association with the gene copy numbers of the other five members of TLRs (*TLR1*, *TLR5*, *TLR6*, *TLR9*, *TLR10*) and BD, VKH syndrome, or AAU ± AS, but no association was detected. As mentioned above, more than 98% of the individuals tested had two copies of these TLR genes.

A recent study from France examining the role of *TLR7* in RA reported that copy numbers of the *TLR7* gene in PBMCs significantly increased with age,[26] The increase they found had a mean amplitude of 20%, spanning 20 to 80 years of age and seen in both male RA patients and in controls. In women, either healthy or having RA, this was not observed, and even an opposite trend was observed.[26] However, we failed to find this phenomenon in our male individuals, nor the females in our study. This may also be due to the fact that the range of ages in our patient group was smaller than that in the French study or that age-linked phenomena may be caused by ethnic differences.

It is not yet clear how *TLR7* affects the predisposition to ocular BD in our Chinese patients. Despite the fact that females carry more copies of the *TLR7* gene, the incidence of BD in our population is much higher in males. The reasons for this discrepancy are unclear but may be due to a higher exposure of males to certain environmental stimuli. Further functional and linkage studies are required to evaluate the exact role of the copy numbers of the *TLR7* gene in ocular BD pathogenesis. The fact that activation of TLRs is dependent on the interaction with its ligands and that the control of the expression of these ligands in BD may depend on as-yet-unknown factors should be considered. A selected population of patients may bias our conclusions because we recruited the ocular BD patients from our ophthalmic center. Recruiting patients from other medical specialities such as a rheumatology department should be carried out in the future to confirm our results. Despite the fact that no association was found concerning the gene copy number and VKH syndrome or AAU ± AS for the other seven TLRs (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR6*, *TLR9*, *TLR10*), this does not exclude the possibility that these TLRs can have an association with BD, VKH syndrome, or AAU ± AS in other ways such as through SNPs. More studies are needed to clarify this issue. Our study was performed in Han Chinese, and whether the same association can be reproduced in other ethnic populations is also a subject for further investigations.

Taken together, our study reports for the first time that a high copy number of the *TLR7* gene indicates risk for ocular BD in a Chinese Han population.

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