Association of *ATG5* Gene Polymorphisms With Behçet's Disease and *ATG10* Gene Polymorphisms With VKH Syndrome in a Chinese Han Population

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Purpose. This study was conducted to explore the association of autophagy-related genes (ATGs) single nucleotide polymorphisms (SNPs) with Behçet's disease (BD) and Vogt-Koyanagi-Harada (VKH) syndrome in a Chinese Han population.

Methods. A two-stage association study was carried out in 940 BD, 1061 VKH, and 2007 healthy controls. Genotyping for genetic variants of 10 autophagy family genes (*ATG5*, *ATG7*, *ATG10*, *ATG16L1*, *IRGM*, *LKKR2*, *ATG2A*, *DAP*, *ULK1*, and *TSC1*) was performed using PCR-restriction fragment length polymorphism (PCR-RFLP) or TaqMan SNP assays. Gene expression was quantified by real-time PCR.

Results. In the cohort of BD patients, we observed that the TT genotype of rs573775/*ATG5* decreased susceptibility to BD ($Pc = 8.35 \times 10^{-6}$, OR = 0.490). In the case of VKH patients, the AC genotype of rs4703863/*ATG10* increased susceptibility to VKH syndrome ($Pc = 9.94 \times 10^{-5}$, OR = 1.444), whereas the A allele and AA genotype of rs4703863 ($Pc = 7.06 \times 10^{-5}$, OR = 0.745; $Pc = 6.34 \times 10^{-6}$, OR = 0.669, respectively) acted as protective factors for VKH. Functional experiments showed an increased *ATG5* expression by LPS stimulated PBMCs in TT cases of rs573775 compared with controls. The level of *ATG5* mRNA in active BD patients not receiving immunosuppression was significantly higher than that in healthy controls.

Conclusions. This study demonstrated an association of *ATG5* rs573775 with BD and *ATG10* rs4703863 with VKH syndrome in a Chinese Han population. Furthermore, a variant of the *ATG5* gene was shown to be correlated with *ATG5* expression.

Keywords: Behçet's disease, VKH syndrome, *ATG5*, *ATG10*

Uveitis is a prevailing eye disease causing blindness worldwide. Behçet's disease (BD) and Vogt-Koyanagi-Harada (VKH) syndrome are two common uveitis entities in Asia. Vogt-Koyanagi-Harada syndrome is a systemic autoimmune disorder that affects pigmented tissues of the body, including the eye.[1] Behçet's disease is a chronic and relapsing autoinflammatory disease that can cause serious complications, including blindness or the rupture of a pulmonary arterial aneurysm.[2] Previous studies have shown that Th1 and Th17 cell responses may play a crucial role in the pathogenesis of these two diseases.[3–7]

Autophagy, a conserved intracellular bulk degradation mechanism, plays vital roles in cells participating in either the innate or adaptive immune system. Autophagy, which is involved in the normal development of B and T lymphocytes, providing metabolic support to proliferating lymphocytes, has been demonstrated as an essential factor in lymphocyte biology.[8–10] Perturbations in the autophagy pathway have been linked to human diseases, including infection and autoimmunity.[11–15] Experimental models of autoimmune disease have shown the requirement of autophagy in dendritic cells (DCs) for the induction of experimental autoimmune encephalomyelitis (EAE).[16]

Recent studies have shown that genetic variants of autophagy-related genes (ATGs) play a role in the predisposition to the development of various autoimmune diseases, such as systemic lupus erythematosus (SLE),[17,18] Crohn's disease,[19–23] and psoriasis.[24,25] To date, the association of ATGs with uveitis has not been investigated and was therefore the purpose of the studies described here.

The study was performed in two well-defined uveitis entities: BD and VKH syndrome. These two entities are relatively common in China, allowing the acquisition of large patient cohorts to obtain sufficient statistical power to study possible genetic associations with these diseases. Our study shows that polymorphisms of *ATG5* are involved in the development of BD, whereas variants of *ATG10* predispose to VKH syndrome.

Methods

Study Population

For this study we recruited 1061 patients with VKH syndrome, 940 BD patients, and 2007 healthy controls from the Zhongshan Ophthalmic Center of Sun Yat-sen University (Guangzhou, China)

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and the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between October 2006 and February 2015. A two-stage case-control association study was carried out. All control subjects were matched ethnically (Han Chinese) and geographically with the patients. Behcet's disease and VKH syndrome were strictly diagnosed based on the criteria of the International Study Group for BD[26] and First International Workshop for VKH syndrome,[27] respectively. The study received the approval of the local ethics research committee and all the investigated subjects provided informed consent before collection of blood. The tenets of the Declaration of Helsinki were adhered to during all procedures of this study.

Single Nucleotide Polymorphism Selection

Our selection of the candidate ATG single nucleotide polymorphisms (SNPs) was based on previously published studies and included only those SNPs showing a positive association with other autoimmune diseases.[17–25,28–30] This resulted in the selection of 16 SNPs in 10 genes, including 4 SNPs (rs9373839, rs573775, rs510432, rs548234) of *ATG5*,[17,18,28,29] 2 SNPs (rs11706903, rs9818393) of *ATG7*,[18,30] 2 SNPs (rs9293293, rs4703863) of *ATG10*,[30] 1 SNP (rs3828309) of *ATG16L1*,[19] 2 SNPs (rs1000113, rs4958847) of Immunity-related GTPase family M protein (*IRGM*),[20] 1 SNP (rs11175593) of Leucine-rich repeat kinase2 (*LRRK2*),[19] 1 SNP (rs17146441) of *ATG2A*,[23] 1 SNP (rs267939) of death-associated protein (*DAP*),[22] 1 SNP (rs12303764) of UNC-51-like kinase 1(*ULK1*),[21] and 1 SNP (rs1076160) of tuberous sclerosis complex1 (*TSC1*).[24,25]

DNA Extraction and Genotyping

Genomic DNA was extracted from blood samples of BD patients, VKH syndrome patients, and healthy controls by using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Samples were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) for rs9373839, rs573775, rs510432, rs548234, rs11706903, rs9818393, rs4703863, rs3828309, rs1000113, rs4958847, rs11175593, and rs17146441. Digestion products were separated on 4% agarose gel and stained with GoldView TM (SBS Genetech, Beijing, China). The rs9293293 (TagMan assay ID: C_30530263_10), rs267939 (TagMan assay ID: C_1973844_10), rs12303764 (TagMan assay ID: C_146323_10), and rs1076160 (TagMan assay ID: C_2536665_10) genotypes were analyzed using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 7500 Real-Time PCR system. The analysis was conducted using TaqMan Genotyper Software (Applied Biosystems). Five percent of all samples selected randomly were sequenced by Majorbio Biotechnology Company (Shanghai, China) to verify the accuracy of genotyping. The success rate of all SNP genotyping ranged from 97.3% to 100%.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque density-gradient centrifugation. Isolated PBMCs (1×10^6 cells per well) were seeded in 24-well plates and cultured in RPMI medium 1640 supplemented with 10% fetal calf serum (Greiner, Wemmel, Belgium), 100 U/mL penicillin, 100 μg/mL streptomycin. To simulate antigen presentation, PBMCs were cultured with a cocktail of anti-CD3 antibody (5 μg/mL; eBioscience, San Diego, CA, USA) and anti-CD28 antibody (1 μg/mL; eBioscience) for 3 days. To simulate an inflammatory signal, PBMCs were cultured with 100 ng/mL lipopolysaccharide (LPS, 100 ng/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) for 24 hours.[31]

Real-Time PCR

Total RNA was extracted from PBMCs with TRIzol (Invitrogen, Carlsbad, CA, USA) followed by reverse transcription using a transcriptase kit. The sequences of the sense and antisense primers were as follows: *ATG5*: 5′-TGTGCTTCGA GATGTGTGGTT-3′ and 5′-ACCAACGTCAAATAGCTGACTC-3′[32]; *ATG10*: 5′-CTTCCCATGGAGGAGGCTTT-3′ and 5′-GGCACTTGGTAGCTACAGGAA-3′. We chose β-actin as the internal reference gene and its expression was detected by the following primers: forward 5′-GGATGCAGAAGGAGATCACTG-3′ and reverse 5′-CGATCCACACGGAGTACTT-3′. The assays were performed on a 7500 real-time instrument (Applied Biosystems). Relative expression levels were calculated using the 2–ΔΔCt method.

Statistical Analysis

Hardy Weinberg equilibrium tests were carried out for all variants for both cases and controls. Statistical analysis was then performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Differences in the genotypic and allelic frequencies were evaluated using a case-control study design and applying a Pearson χ^2 test; *P* values were assessed with the χ^2 test or Fisher's exact test and *P* values were corrected (Pc) for multiple comparisons with the Bonferroni correction method by multiplying with the number of analyses performed. The number of independent comparisons is 48. To investigate whether associations could be explained by either additive codominant, dominant, allelic, or recessive models, the data of rs573775 and rs4703863 genotype frequencies were analyzed by univariate logistic regression and multivariate logistic regression. The nonparametric Mann-Whitney test was used to compare *ATG5*, *ATG10* expression among three genotype groups.

Results

Clinical Features of Patients With BD and VKH Syndrome

The clinical characteristics, sex, and age of the enrolled BD and VKH patients with uveitis are displayed in Table 1. In addition, age and sex distribution of controls are presented. The genotype frequencies of the 16 SNPs did not deviate from the Hardy–Weinberg equilibrium in the controls.

Allele and Genotype Frequencies of SNPs in Patients and Controls in the First-Stage Study

Sixteen SNPs were genotyped in 384 VKH, 384 BD, and 576 controls for the first-stage study. The frequency of the rs573775/*ATG5* TT genotype was significantly lower in BD patients ($Pc = 6.38 \times 10^{-4}$, odds ratio [OR] = 0.351) (Table 2). In the case of rs4703863/*ATG10*, an increased frequency of the AC genotype was observed in VKH patients ($Pc = 2.07 \times 10^{-2}$, OR = 1.606), whereas a decreased frequency of the A allele and AA genotype ($Pc = 1.25 \times 10^{-2}$, OR = 0.676; $Pc = 3.6 \times 10^{-3}$, OR = 0.591, respectively) was found (Table 3). We did not find a significant association of the other 14 SNPs with VKH or BD (Supplementary Tables S1, S2).

Allele and Genotype Frequencies of SNPs in Patients and Controls in the Second-Stage Study and Combined Study

Because rs573775/*ATG5* and rs4703863/*ATG10* showed a significant association with BD and VKH, respectively, in the

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Table 1. Clinical Characteristics, Sex, and Age of BD and VKH Patients With Uveitis

Clinical Features	Total	%
Patients with BD		
Mean age ± SD	35.1 ± 9.5	
Male	790	84.0
Female	150	16.0
Uveitis	940	100
Oral ulcer	940	100
Genital ulcer	538	57.2
Skin lesion	709	75.4
Arthritis	161	17.1
Pathergy reaction	229	24.4
Patients with VKH		
Mean age ± SD	33.4 ± 8.9	
Male	570	53.7
Female	491	46.3
Uveitis	1061	100
Headache	537	50.6
Tinnitus	420	39.6
Vitiligo	283	26.7
Alopecia	230	21.7
Gray hair	257	24.2
Controls		
Mean age ± SD	36.3 ± 12.3	
Male	1092	54.4
Female	915	45.6

first stage, we subsequently tested another set of 556 BD patients, 677 VKH patients, and 1431 controls in the second-stage study. The result again demonstrated a significantly lower frequency of the rs573775/*ATG5* TT genotype in BD patients ($Pc = 4.8 \times 10^{-2}$, OR = 0.588) (Table 2). In VKH patients, decreased frequencies of the rs4703863/*ATG10* A allele and AA genotype ($Pc = 4.8 \times 10^{-2}$, OR = 0.777; $Pc = 1.08 \times 10^{-2}$, OR = 0.708, respectively) were found, whereas an increased frequency of the AC genotype was observed ($Pc = 4.8 \times 10^{-2}$, OR = 1.380) (Table 3). When adding up the data of the first- and second-stage study (combined study), we were able to confirm the association of rs573775/*ATG5* with BD patients (TT genotype: $Pc = 8.35 \times 10^{-6}$, OR = 0.490) (Table 2) and rs4703863/*ATG10* with VKH patients (AA genotype: $Pc = 6.34 \times 10^{-6}$, OR = 0.669; AC genotype: $Pc = 9.94 \times 10^{-5}$, OR = 1.444; A allele: $Pc = 7.06 \times 10^{-5}$, OR = 0.745) (Table 3). We subsequently investigated whether the association with rs573775 and rs4703863 behaved as dominant, recessive, or codominant using univariate and multivariate logistic regression analysis. The rs573775/*ATG5* T allele association with BD behaved as a recessive model (Table 4). The Rs4703863/*ATG10* C allele association with VKH behaved as a dominant model (Table 5).

Stratified Analysis of rs573775 With Main Clinical Features of BD and rs4703863 With VKH Syndrome

A stratified analysis was performed to examine the association of rs573775 with the main clinical features of BD and rs4703863 with the main clinical features of VKH disease. The main clinical manifestations of VKH consisted of headache, tinnitus, vitiligo, gray hair, and alopecia. The main clinical features of BD included genital ulcer, skin lesions, arthritis, and the so-called pathergy reaction. No significant association was found for the individual extraocular manifestations of either BD or VKH with the tested SNPs (Supplementary Tables S3, S4).

The Influence of rs573775 on Autophagy-Related Gene 5 and rs4703863 on Autophagy-Related Gene 10 Expression

The aforementioned data showed that genetic polymorphisms of *ATG5* and *ATG10* are associated with susceptibility to BD and VKH, respectively. We subsequently investigated whether different genotypes could have an effect on the expression of *ATG5* and *ATG10* in PBMCs under normal or inflammatory conditions from 47 healthy controls by real-time PCR. No significant association was found in *ATG5* gene expression

Table 2. Main Effect of *ATG5*/rs573775 on BD Risk

Genotype	BD, *n* (%)	Controls, *n* (%)	*P* Value	Pc Value	OR (95% CI)
Stage 1	*n* = 384	*n* = 576			
TT	22 (5.7)	85 (14.8)	1.33×10^{-5}	6.38×10^{-4}	0.35 (0.22–0.57)
CT	211 (54.9)	263 (45.7)	0.05	NS	1.45 (1.12–1.88)
CC	151 (39.3)	228 (39.6)	0.94	NS	0.99 (0.76–1.29)
T allele	255 (33.2)	433 (37.6)	0.05	NS	0.83 (0.68–1.00)
C allele	513 (66.8)	719 (62.4)	0.05	NS	1.21 (1.00–1.47)
Stage2	*n* = 556	*n* = 1431			
TT	49 (8.8)	202 (14.1)	1.00×10^{-3}	4.80×10^{-2}	0.59 (0.42–0.82)
CT	286 (51.4)	697 (48.7)	0.27	NS	1.12 (0.92–1.36)
CC	221 (39.7)	532 (37.2)	0.29	NS	1.12 (0.91–1.36)
T allele	384 (34.5)	1101 (38.5)	0.21	NS	0.84 (0.73–0.98)
C allele	728 (65.5)	1761 (61.5)	0.21	NS	1.19 (1.03–1.37)
Combined	*n* = 940	*n* = 2007			
TT	71 (7.6)	287 (14.3)	1.74×10^{-7}	8.35×10^{-6}	0.49 (0.37–0.64)
CT	497 (52.9)	960 (47.8)	0.01	NS	1.22 (1.05–1.43)
CC	372 (39.5)	760 (37.9)	0.38	NS	1.08 (0.92–1.26)
T allele	639 (34.0)	1534 (38.2)	0.02	NS	0.83 (0.74–0.93)
C allele	1241 (66.0)	2480 (61.8)	0.02	NS	1.20 (1.07–1.35)

CI, confidence interval.

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Table 3. Main Effects of *ATG10*/rs4703863 on VKH Risk

Genotype	VKH, *n* (%)	Controls, *n* (%)	*P* Value	Pc Value	OR (95% CI)
Stage 1	$n = 384$	$n = 576$			
AA	187 (48.7)	355 (61.6)	7.51×10^{-5}	3.60×10^{-3}	0.59 (0.46–0.77)
AC	174 (45.3)	196 (34.0)	4.32×10^{-4}	2.07×10^{-2}	1.61 (1.23–2.09)
CC	23 (6.0)	25 (4.3)	0.25	NS	1.40 (0.79–2.51)
A allele	548 (71.4)	906 (78.6)	2.61×10^{-4}	1.25×10^{-2}	0.68 (0.55–0.84)
C allele	220 (28.6)	246 (21.4)	2.61×10^{-4}	1.25×10^{-2}	1.48 (1.20–1.83)
Stage2	$n = 677$	$n = 1431$			
AA	342 (50.5)	845 (59.0)	2.26×10^{-4}	1.08×10^{-2}	0.71 (0.59–0.85)
AC	291 (43.0)	508 (35.5)	1.00×10^{-3}	4.80×10^{-2}	1.38 (1.15–1.66)
CC	44 (6.5)	78 (5.5)	0.34	NS	1.21 (0.82–1.77)
A allele	975 (72.0)	2198 (76.8)	1.00×10^{-3}	4.80×10^{-2}	0.78 (0.67–0.90)
C allele	379 (28.0)	664 (23.2)	1.00×10^{-3}	4.80×10^{-2}	1.29 (1.11–1.49)
Combined	$n = 1061$	$n = 2007$			
AA	529 (49.9)	1200 (59.8)	1.32×10^{-7}	6.34×10^{-6}	0.67 (0.58–0.78)
AC	465 (43.8)	704 (35.1)	2.07×10^{-6}	9.94×10^{-5}	1.44 (1.24–1.68)
CC	67 (6.3)	103 (5.1)	0.17	NS	1.24 (0.91–1.71)
A allele	1523 (71.8)	3104 (77.3)	1.47×10^{-6}	7.06×10^{-5}	0.75 (0.66–0.84)
C allele	599 (28.2)	910 (22.7)	1.47×10^{-6}	7.06×10^{-5}	1.34 (1.19–1.51)

Table 4. Logistic Regression Analysis of the Risk of BD Patients With *ATG5*/rs573775 in Additive Codominant, Dominant, Allelic, and Recessive Models

Model	Genotype	Control, $n = 2007$, *n* (%)	Case, $n = 940$, *n* (%)	Univariate Logistic Regression		Multivariate Logistic Regression*	
				OR (95% CI)	*P*†	OR (95% CI)	*P*†
Additive				0.82 (0.73–0.93)	1.20×10^{-3}	0.84 (0.74–0.95)	4.50×10^{-3}
Codominant	CC	760 (37.9)	372 (39.6)	Reference		Reference	
	CT	960 (47.8)	497 (52.9)	1.06 (0.90–1.25)	0.50	1.11 (0.94–1.32)	0.24
	TT	287 (14.3)	71 (7.6)	0.51 (0.38–0.67)	3.39×10^{-6}	0.51 (0.38–0.68)	7.94×10^{-6}
Dominant	CC	760 (37.9)	372 (39.6)	Reference		Reference	
	CT+TT	1247 (62.1)	568 (60.4)	0.93 (0.79–1.09)	0.38	0.97 (0.82–1.14)	0.70
Recessive	CC+CT	1720 (85.7)	869 (92.5)	Reference		Reference	
	TT	287 (14.3)	71 (7.6)	0.49 (0.37–0.64)	2.75×10^{-7}	0.48 (0.36–0.63)	2.86×10^{-7}
Allelic	C	1720 (58.0)	869 (60.5)	Reference		Reference	
	T	1247 (42.0)	568 (39.5)	0.90 (0.79–1.03)	0.11	0.91 (0.80–1.05)	0.19

* The age and sex were adjusted in the multivariate logistic regression model.
† The hypothesis test was performed using the Wald χ^2 test.

Table 5. Logistic Regression Analysis of the Risk of VKH Patients With *ATG10*/rs4703863 in Additive Codominant, Dominant, Allelic, and Recessive Models

Model	Genotype	Control, $n = 2007$, *n* (%)	Case, $n = 1061$, *n* (%)	Univariate Logistic Regression		Multivariate Logistic Regression*	
				OR (95% CI)	*P*†	OR (95% CI)	*P*†
Additive				1.36 (1.20–1.53)	1.19×10^{-6}	1.34 (1.18–1.51)	4.08×10^{-6}
Codominant	AA	1200 (59.8)	529 (49.9)	Reference		Reference	
	AC	704 (35.1)	465 (43.8)	1.50 (1.28–1.75)	3.46×10^{-7}	1.48 (1.27–1.73)	8.73×10^{-7}
	CC	103 (5.1)	67 (6.3)	1.48 (1.07–2.04)	0.02	1.43 (1.03–1.98)	0.03
Dominant	AA	1200 (59.8)	529 (49.9)	Reference		Reference	
	AC+CC	807 (40.2)	532 (50.1)	1.50 (1.29–1.74)	1.41×10^{-7}	1.48 (1.27–1.72)	4.53×10^{-7}
Recessive	AA+AC	1904 (94.9)	994 (93.7)	Reference		Reference	
	CC	103 (5.1)	67 (6.3)	1.25 (0.91–1.71)	0.17	1.21 (0.88–1.67)	0.24
Allelic	A	1904 (70.2)	994 (65.1)	Reference		Reference	
	C	807 (29.8)	532 (34.9)	1.26 (1.11–1.44)	6.00×10^{-4}	1.25 (1.09–1.43)	1.10×10^{-3}

* The age and sex were adjusted in the multivariate logistic regression model.
† The hypothesis test was performed using the Wald χ^2 test.

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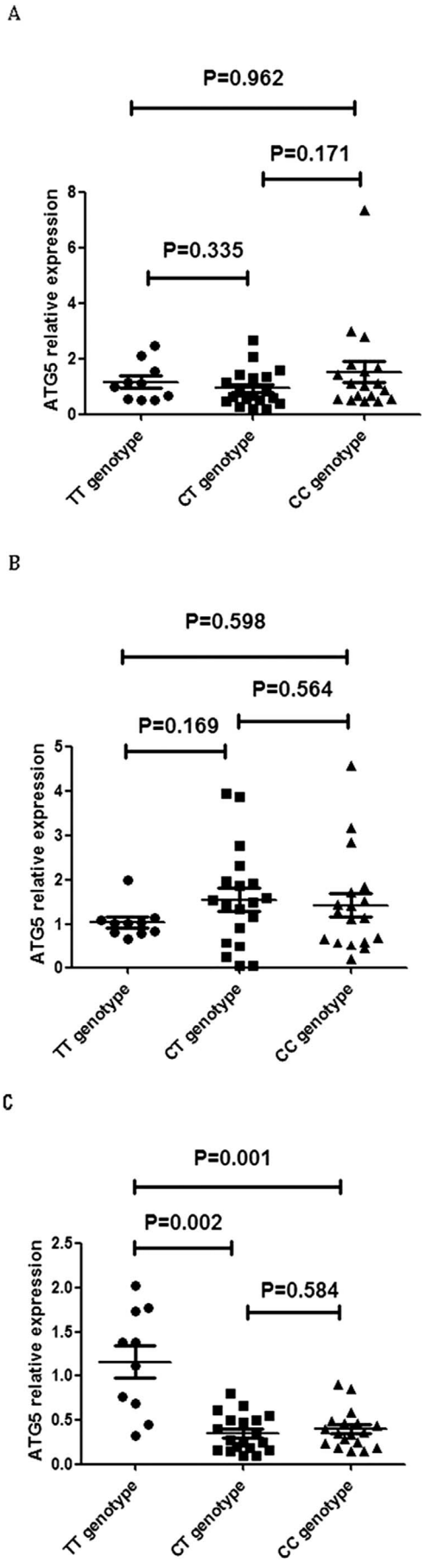

between the various genotypes when PBMCs were left unstimulated or were stimulated with a cocktail of anti-CD3/CD28 antibodies (Figs. 1A, 1B). Following stimulation with LPS, carriers with the TT genotype in SNP, rs573775 had a higher *ATG5* mRNA expression compared with individuals carrying the CT ($P = 0.002$) or CC ($P = 0.001$) genotype (Fig. 1C). Furthermore, the level of *ATG5* mRNA in PBMCs obtained from active BD patients was significantly higher than that observed in healthy controls in ($P = 0.029$) (Fig. 2). No effect on *ATG10* mRNA expression was observed for rs4703863 among different genotypes regardless of whether PBMCs had been stimulated or not (Supplementary Fig. S1). Additionally, no significant difference of *ATG10* mRNA expression was observed between active VKH patients and healthy controls (Supplementary Fig. S2).

Discussion

We investigated the association of 16 autophagy-related gene variants with BD and VKH and showed that the AA genotype and A allele of *ATG10*/rs4703863 had a protective effect on VKH syndrome, whereas the TT genotype of *ATG5*/rs573775 protected from developing BD. Functional studies showed that individuals with the TT genotype of rs573775 had a higher *ATG5* expression as compared with individuals carrying the CT or CC genotype. Our study thus adds uveitis to the list of immune disorders, whereby autophagy-related genes are involved.[33]

Autophagy, which is involved in the activation of innate and adaptive immune responses, plays a key role in dead-cell clearance, self-antigen presentation, and in the regulation of lymphocyte development, survival, and proliferation.[10,34–36]

The *ATG10*, which is an autophagic E2 enzyme, interacts with *ATG7* to receive an ubiquitin-like molecule *ATG12*, and is also involved in the *ATG12–ATG5* conjugation reaction.[37] The *ATG10* has been shown to play a role in the proliferation and invasion of cancer cells[38] and an association of genetic variants in *ATG10* with breast cancer has been reported.[39] A possible role of *ATG10* in autoimmune or autoinflammatory disease has not yet been reported. During our genome-wide association studies (GWAS) for VKH, we discovered 2 SNPs in *ATG10* that may confer risk to VKH ($5 \times 10^{-8} < P < 0.05$).[30] Although these SNPs did not reach the GWAS *P*-value threshold, we decided to include them in the current study with a larger patient sample size. The protective effect of the AA genotype of *ATG10*/rs4703863 could be confirmed but is modest, whereby 60% of controls and 50% of VKH patients carry this genotype. Furthermore, due to the very low frequency of the CC genotype both in patients and controls, we could not detect a positive predisposing effect of the CC genotype of *ATG10*/rs4703863 in VKH ($Pc > 0.05$, OR = 1.246); however, we did observe that the C allele of *ATG10*/rs4703863 conferred risk to VKH ($Pc = 7.06 \times 10^{-5}$, OR = 1.342). How *ATG10* affects predisposition to VKH is not clear, and functional studies did not show an effect of the *ATG10* genotypes on mRNA expression by PBMCs. Comparison of *ATG10* mRNA expression between active VKH patients and

Figure 1. The influence of various rs573775 genotypes on the expression of *ATG5*. (**A**) Expression of *ATG5* in nonstimulated PBMCs from normal controls carrying different genotypes of rs573775 (TT = 10, CT = 19, CC = 18). (**B**) Expression of *ATG5* in anti-CD3/CD28 antibodies stimulated PBMCs from healthy controls carrying different genotypes of rs573775 (TT = 10, CT = 19, CC = 18). (**C**) Expression of *ATG5* in LPS-stimulated PBMCs from healthy controls carrying different genotypes of rs573775 (TT = 10, CT = 19, CC = 18).

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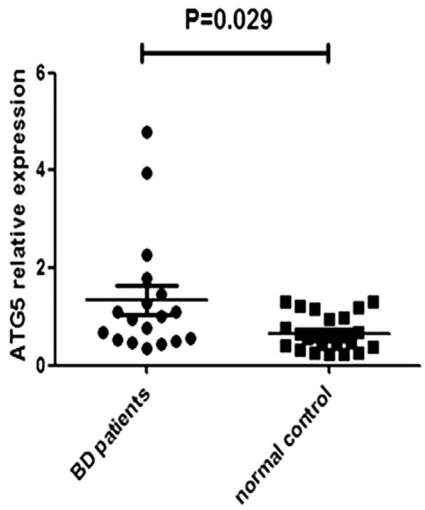

FIGURE 2. The expression of *ATG5* in active BD patients and healthy controls. The mRNA expression of *ATG5* in active BD patients not receiving immunosuppression and healthy controls (BD patients = 18, healthy controls = 24).

healthy controls also did not show a significant difference, and further studies are needed to elucidate the exact role of *ATG10* in the development of VKH.

The *ATG5*, which is necessary for antigen presentation,[40] can lead to increased viral clearance.[41] The autophagy machinery also can be hijacked to increase viral replication.[42] It was reported that *ATG5* independently influences life and death decisions of the cell by both "autophagic" cell death and apoptotic death pathways.[43] Various studies have provided evidence showing that genetic variants of *ATG5* are associated with immune system disorders such as systemic sclerosis, SLE, and asthma.[17,18,28,29] Our finding that *ATG5* is associated with ocular BD has not yet been reported earlier, and our study is the first showing a protective effect of the TT genotype of *ATG5*/rs573775 in an autoinflammatory disease. An opposite result was reported in female SLE patients of European ancestry, whereby the T allele of *ATG5*/rs573775 was identified as a possible risk allele (T allele: $P = 1.36 \times 10^{-7}$, OR = 1.19).[17] In this study, 28% of controls and 32% of female SLE patients carried the T allele. The association of SLE with the *ATG5* rs573775 T allele could not be confirmed in a Spanish group of SLE patients but susceptibility became apparent when a functional IL-10 genotype was included in the analysis.[44] Of interest was the observation that serum IL-10 levels were higher in the *ATG5* TT genotype SLE patients compared with the other genotypes. A Chinese study confirmed the association between *ATG5* gene variants and SLE, but they did not study the rs573775 allele.[18]

An association between *ATG5* genetic variants and systemic sclerosis was recently reported but no association was found for many other autoimmune diseases such as rheumatoid arthritis, celiac disease, psoriasis, juvenile idiopathic arthritis, primary biliary cirrhosis, narcolepsy, and autoimmune thyroid disease.[28]

The discrepancies between *ATG5* rs573775 association between SLE and BD might be due to differences in the pathogenesis of the two diseases. Behcet disease is considered a chronic autoinflammatory disease involving an increased Th1 and Th17 cell response, whereas SLE is an autoimmune disease characterized by failure of multiple tolerance checkpoints, leading to the escape and proliferation of autoreactive B cells.[45,46]

How the *ATG5* genotype protects against BD development is not clear. Enhanced *ATG5* mRNA expression might be associated with a higher level of autophagosome formation, although additional experiments are needed to support this hypothesis. An exaggerated immune response against microbial pathogens has been implicated in the pathogenesis of BD[47] and the role of autophagy in the defense against pathogens might provide the link explaining the association between genetic *ATG5* variants and BD development. A recent in vivo study showed that *ATG5* was involved in LPS-induced inflammatory response in a mice macrophage polarization and showed that *ATG5* operated as a negative regulatory feedback mechanism to prevent an inflammatory reaction.[48] The data were in agreement with our study, where we observed protection against BD with a genotype associated with higher *ATG5* expression. Our approach to study the effect of *ATG5* expression in PBMCs stimulated with LPS is a novel approach and awaits confirmation by other groups.

We only examined the effect of rs573775 in healthy controls, because studies in patients are confounded by the fact that the inflammatory response in patients is extremely heterogeneous and in view of the fact that most are treated by immunosuppressive drugs. We did study a small group of untreated BD patients and showed that active BD patients had a remarkably higher expression of *ATG5* as compared with healthy controls. On one hand, a high genetically predisposed production of *ATG5* mRNA will protect against BD, whereas disease activity is also associated with a higher *ATG5* mRNA expression. This finding is consistent with previous studies in patients with other autoimmune diseases whereby it was shown that expression of *ATG5* was increased during EAE in mice and in subjects with multiple sclerosis.[49] Expression of *ATG5* mRNA was also shown to be upregulated in human nasal epithelial cells during an acute asthma attack.[29]

It is worthwhile to point out that, as mentioned above, our group previously carried out GWAS in both conditions, and no significant association was observed at that time for the ATGs.[30,50] The samples and methods involved in the BD GWAS, VKH GWAS, and the current study were different and the observed discrepancies may be explained as follows. First, the cohorts of patients for our GWAS were not the same as used in the present study. In our earlier BD GWAS study, all patients and healthy controls were recruited before 2012, whereas in the present study, the patients and healthy controls were collected between 2006 and 2015. The cohort of patients for the VKH GWAS were recruited from multiple ophthalmic centers in China, whereas the VKH patients included in the present study came from our own departments in Guangzhou and Chongqing. The fact that the ATG associations reported in the present study were not apparent in the GWAS may be due to the following: (1) the GWAS analysis uses a $P < 5 \times 10^{-8}$ as statistically significant, and this stringency was higher than what we used in our current study; (2) GWAS is based on the assumption of indirect association mapping using reference SNPs in linkage disequilibrium with the phenotype of interest. It is possible that the SNPs used in our current study are not in LD with these GWAS reference loci, possibly due to a location in an area of high recombination. Further studies are needed to clarify this issue.

Our study has several limitations. The BD patients enrolled were predominantly male and further studies should be performed in a sex-matched population. Beyond that, we chose only previously reported loci, which were associated with autoimmune diseases and it cannot be ruled out that other SNPs in autophagy-related genes can be associated with VKH syndrome and BD. Moreover, we studied only two widespread uveitis entities and it is possible that autophagy-

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related gene associations may be present in other types of intraocular inflammation or other ethnic populations.

Conclusions

Taken together, this is the first report showing that an *ATG5* variant is associated with BD and that an *ATG10* variant predisposes to VKH syndrome.

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