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Toll-like receptor 3 polymorphism is not associated with neovascular age-related macular degeneration and polypoidal choroidal vasculopathy in the Chinese

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ABSTRACT. Toll-like receptor 3 (TLR3) variants in mainland northern Chinese patients with polypoidal choroidal vasculopathy (PCV) and neovascular age-related macular degeneration (nAMD) were investigated. The complete genes of TLR3, including all exons and the promoter region, were assessed using direct sequencing technology of 284 unrelated mainland northern Chinese individuals: 96 nAMD patients, 92 PCV patients, and 96 controls. Six single

nucleotide polymorphisms were identified: rs5743303, rs5743305, rs5743312, rs3775291, rs3775290, and rs6830345. The distribution of TLR3 genotypes for nAMD and PCV was not significantly different compared with normal controls. This study indicates that the *TLR3* gene polymorphism is not associated with nAMD and PCV in northern Chinese patients.

Key words: Neovascular age-related macular degeneration; TLR3; Polypoidal choroidal vasculopathy; Single nucleotide polymorphism

INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of severe visual impairment in the world. It is characterized by chronic and progressive degeneration of photoreceptors, the underlying retinal pigment epithelium, Bruch's membrane, and possibly the choriocapillaris in the macula and affects more than 10 million people worldwide (Friedman et al., 2004). Advanced AMD is of 2 forms: geographic atrophy (GA) of the retinal pigment epithelium and overlying photoreceptors (called advanced "dry" AMD) and choroidal neovascularization (CNV, called "wet" AMD or neovascular AMD, nAMD). Polypoidal choroidal vasculopathy (PCV) is a major cause of serosanguinous maculopathy in elderly Chinese and Japanese patients with choroidal neovascular anomaly (Yannuzzi et al., 1999; Wen et al., 2004). PCV has been described as a separate clinical entity, differing from AMD and other diseases associated with subretinal neovascularization (Ciardella et al., 2004), and it remains controversial whether or not PCV represents a subtype of nAMD. The incidences of PCV in the Chinese and Japanese populations with nAMD have been reported at 24.5 and 54.7%, respectively, much higher than in Caucasians (Lafaut et al., 2000; Ladas et al., 2004; Wen et al., 2004; Maruko et al., 2007).

Familial (Klaver et al., 1998; Smith and Mitchell, 1998) and twin studies (Klein et al., 1994; Meyers et al., 1995) implicated the role of genetic predisposition of AMD (Katta et al., 2009). Genome-wide association studies identified the complement factor H (CFH) gene on chromosome 1q32 as a susceptibility gene for AMD (Edwards et al., 2005; Klein et al., 2005). Later, a single nucleotide polymorphism (SNP; rs10490924, c.205G>T, A69S) in the age-related maculopathy susceptibility 2 (ARMS2) gene on chromosome 10q26 was reported to be strongly associated with AMD (Rivera et al., 2005). PCV, a disease sharing similar phenotypes with exudative AMD, was reported to be associated with *CFH* and *ARMS2*/*HTRA1* in Caucasians (Lima et al., 2010), Japanese (Kondo et al., 2007; Kondo et al., 2009; Nakanishi et al., 2010), and Singapore Chinese (Lee et al., 2008) populations.

Previous reports described the first genetic variant, Toll-like receptor 3 (*TLR3)* gene rs3775291, linked to slightly higher risk for severe "dry" AMD, one of the two advanced forms of AMD. The researchers specifically targeted genes for Toll-like receptors, proteins that recognize pathogens and signal the immune system (Kaiser, 2008; Yang et al., 2008). TLR3 recognizes double-stranded RNA (dsRNA) from viruses and induces infected cells to undergo apoptosis (Zhou et al., 2011) Kleinman et al. (2008) demonstrated that siRNA-mediated signaling through TLR3 can suppress experimentally induced nAMD independent of the gene targeted by the siRNA. A proposed mechanism for the observed anti-angiogenic function of TLR3 is activation of IL-12 and IFN-γ, 2 mediators that inhibit neovascularization

(Kleinman et al., 2008). A second report further demonstrated that both hemangiogenesis and lymphangiogenesis could be suppressed via TLR3 signaling in models of neovascularization induced by corneal sutures and ischemia (Cho et al., 2009a). Moreover, researchers found the association between TLR3 and human CNV membranes and that TLR3 may be upregulated during CNV formation (Maloney et al., 2010). In summary, these studies suggest a suppressive role of TLR3 in pathological neovascularization. However, some studies reported that this *TLR3* SNP was not associated with nAMD in Caucasian samples (Allikmets et al., 2009; Cho et al., 2009b). It remains uncertain whether TLR3 variants play a role in the molecular pathogenesis of nAMD in the Chinese.

Because the genetic architecture underlying complex disorders such as nAMD and PCV may have population-specific differences, TLR3 polymorphism may be associated with PCV in the northern Chinese. The aim of this study was therefore to examine this association in northern Chinese subjects with nAMD and PCV.

MATERIAL AND METHODS

Subjects

Two hundred and eighty-four unrelated northern Chinese were studied (Table 1); 96 patients had nAMD [mean age ± standard deviation (SD), 70.3 ± 8.8 years; ratio of men to women, 64.6:35.4] and 92 patients had PCV (mean age ± SD, 69.5 ± 9.4 years; ratio of men to women, 52.2:47.8). For controls, 96 individuals without ARM were studied (mean age ± SD, 67 ± 9.5 years; ratio of men to women, 44.8:55.2). These individuals were recruited to the Department of Ophthalmology at the Peking University People's Hospital. The study was approved by the Ethics Committee of Peking University People's Hospital. An informed consent process was established following the guidelines of the Helsinki Declaration, and consent forms were signed by all subjects. All subjects received a standard ophthalmic examination, including visual acuity measurement, slit-lamp biomicroscopy, and dilated fundus examination, which were performed by a retinal specialist. All cases with AMD and PCV underwent fluorescein angiography, optic coherence tomography, and indocyanine green angiograms with HRA2 (Heidelberg Engineering, Heidelberg, Germany). Diagnosis of nAMD and ARM was defined by the International Classification System for ARM (Bird et al., 1995). The diagnosis of PCV was based on indocyanine green angiography results, which showed a branching vascular network that terminated in aneurysmal enlargements, that is, polypoidal lesions. Eyes with other macular abnormalities, such as pathologic myopia, idiopathic CNV, presumed ocular histoplasmosis, angioid streaks, and other secondary CNV, were excluded. Normal controls were defined as no clinical evidence of early or late AMD in either eye or any other eye diseases except mild age-related cataract. Subjects with severe cataracts were excluded from the study.

Table 1. Characteristics of the study population.

	nAMD	PCV	Controls	P
Total	96	92	96	
Males [N (%)]	62 (64.6)	48 (52.2)	43 (44.8)	>0.05
Females [N (%)]	34 (35.4)	44 (47.8)	53 (55.2)	
Mean age (±SD; years)	70.3 ± 8.8	69.5 ± 9.4	67 ± 9.5	0.08

nAMD = neovascular age-related macular degeneration; PCV = polypoidal choroidal vasculapathy.

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from venous blood leukocytes with a genomic extraction kit (eBios Biotechnology Co., Beijing, China). A PCR amplification kit was purchased from Dingguo Biotechnology (Beijing, China) Co. The PCR primers were designed using Primer 3 and synthesized by Invitrogen Corporation (Shanghai, China). A PCR amplification was performed in 50-µL reactions containing 50 ng genomic DNA, 20 µM of each primer, 32 µL ddH_2O, 2.5 mM dNTPs, 2.0 U Taq, and 5 µL 10X PCR buffer. Thermocycling was performed with an initial denaturation step at 94°C for 5 min; followed by 10 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 45 s; followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 45 s; followed by a final extension step at 72°C for 3 min. The PCR products were assessed by 1.0% agarose gel electrophoresis. DNA bands with correct sizes were purified with a 96-well PCR purification kit (Millipore, USA).

Genotyping by sequencing

All purified PCR products were directly sequenced with an ABI 3730XL DNA sequencer. Variants of the *TLR3* gene were identified with the ABI automatic allele calling software. Genotyping had 99% completeness and 99% accuracy as determined by random re-sequencing of 10% of the samples.

Statistical analysis

The statistical significance of the distribution of the alleles and genotypic frequencies among the nAMD patients, PCV patients, and control subjects was determined by the Pearson chi-square test. Odds ratios and 95% confidence intervals were calculated using SPSS v.18 (SPSS, Chicago, IL, USA), and the Bonferroni correction method was used to correct for multiple testing. P values less than 0.05 were considered to be statistically significant.

RESULTS

The complete gene of *TLR3*, including all exons and the promoter region, were sequenced. Six SNPs were identified: rs5743303, rs5743305, rs5743312, rs3775291, rs3775290, and rs6830345. Two of the SNPs were in the promoter region, 2 of the SNPs were in introns, and the remaining 2 SNPs were in exon 4. However, the allelic and genotypic distributions of the *TLR3* SNP genotypes for PCV and nAMD were not significantly different from those in normal controls, when examined using genotypic, additive, dominant, and recessive models. We also found no statistically significant association before adjustment for age and gender, or when both nAMD and PCV were combined in the analysis (Table 2).

DISCUSSION

TLRs comprise a family of 10 to 12 type I integral membrane receptor paralogs that are expressed in ocular tissues, inducing apoptosis in response to dsRNA, a molecular pattern associated with viral infection that is produced by most viruses at some point during their

Table 2. Polymorphisms in the *TLR3* gene lesion for distribution and genotypes (**A**) and distribution and alleles (**B**) in neovascular age-related macular degeneration (nAMD), polypoidal choroidal vasculopathy (PCV), and controls in the northern Chinese population.

A.

Marker	Relative position	Risk allele	Wild-type allele	Risk allele frequency				P value and OR (95%CI)		
				nAMD	PCV	Controls		nAMD-control	PCV-control	nAMD-PCV
rs5743303	Promoter	T	A	0.16	0.19	0.27	P values	0.08	0.21	0.49
							OR (95%CI)	1.93 (0.92-4.06)	1.60 (0.76-3.34)	1.21 (0.71-2.07)
rs5743305	Promoter	A	T	0.30	0.30	0.20	P values	0.15	0.15	0.96
							OR (95%CI)	0.56 (0.25-1.24)	0.56 (0.25-1.25)	0.99 (0.62-1.57)
rs5743312	Intron 3	T	C	0.21	0.24	0.29	P values	0.48	0.72	0.59
							OR (95%CI)	1.31 (0.62-2.77)	1.15 (0.54-2.41)	1.15 (0.70-1.89)
rs3775291	Exon 4	T	C	0.23	0.31	0.27	P values	0.99	0.29	0.17
							OR (95%CI)	0.99 (0.42-2.34)	0.66 (0.30-1.44)	0.51 (0.84-2.72)
rs3775290	Exon 4	T	C	0.29	0.32	0.31	P values	0.780	0.94	0.59
							OR (95%CI)	1.10 (0.55-2.20)	0.97 (0.49-1.93)	1.13 (0.72-1.78)
rs6830345	Intron 4	C	T	0.86	0.87	0.85	P values	0.94	0.82	0.83
							OR (95%CI)	0.96 (0.39-2.38)	0.90 (0.36-2.24)	1.07 (0.59-1.94)

$P < 0.05$ is considered to be statistically significant. OR (95%CI) = odds ratio and 95% confidence interval are given for the risk allele compared with the WT allele.

B.

SNP	Genotype distribution (%)				nAMD *vs* controls			PCV *vs* controls			nAMD *vs* PCV		
		nAMD	PCV	Controls		Genotypic	Homozygous	Heterozygous	Genotypic	Homozygous	Heterozygous	Genotypic	Homozygous	Heterozygous
rs5743303	AA	67 (69.8)	59 (65.6)	56 (58.3)	P values	0.07	0.02	0.68	0.20	0.08	0.92	0.77	0.56	0.61
	AT	27 (28.1)	28 (31.1)	28 (29.2)	OR (95%CI)		0.14 (0.02-0.91)	0.81 (0.29-2.22)		0.24 (0.04-1.30)	0.95 (0.35-2.61)		0.59 (0.10-3.63)	0.85 (0.45-1.60)
	TT[$]	2 (2.1)	3 (3.3)	12 (12.5)										
rs5743305	TT	41 (50.0)	42 (48.8)	56 (58.3)	P values	0.23	NA	0.69	0.26	NA	0.55	0.90	0.79	0.78
	TA	32 (39.0)	36 (41.9)	40 (31.7)	OR (95%CI)		NA	1.21 (0.47-3.16)		NA	1.33 (0.52-3.44)		1.15 (0.41-3.28)	0.91 (0.48-1.73)
	AA[$]	9 (11.0)	8 (9.3)	0										
rs5743312	CC	60 (65.2)	52 (59.8)	56 (58.3)	P values	0.71	0.41	0.96	0.56	0.42	0.63	0.67	0.99	0.38
	CT	25 (27.2)	29 (33.3)	24 (25.0)	OR (95%CI)		0.54 (0.13-2.37)	0.97 (0.34-2.82)		0.54 (0.12-2.43)	1.30 (0.45-3.75)		1.01 (0.32-3.20)	0.75 (0.39-1.43)
	TT[$]	7 (7.6)	6 (6.9)	16 (16.7)										
rs3775291	CC	27 (58.7)	37 (45.7)	52 (54.2)	P values	0.99	0.98	0.97	0.53	0.50	0.31	0.35	0.35	0.20
	CT	17 (37.0)	38 (46.9)	36 (37.5)	OR (95%CI)		0.96 (0.80-11.61)	1.02 (0.35-2.98)		2.11 (0.23-19.20)	1.67 (0.62-4.49)		0.46 (0.09-2.44)	0.61 (0.29-1.31)
	TT[$]	2 (4.3)	6 (7.4)	8 (0.8)										
rs3775290	CC	44 (49.4)	44 (48.4)	36 (37.5)	P values	0.13	NA	0.16	0.60	NA	0.13	0.63	0.39	0.86
	CT	38 (42.7)	36 (39.6)	60 (62.5)	OR (95%CI)		NA	0.52 (0.20-1.32)		NA	0.49 (0.19-1.25)		0.64 (0.23-1.79)	1.06 (0.57-1.96)
	TT[$]	7 (7.9)	11 (12.0)	0										
rs6830345	TT	3 (3.3)	0	0	P values	0.53	NA	NA	0.81	NA	NA	0.18	NA	NA
	TC	20 (21.7)	24 (26.7)	28 (29.2)	OR (95%CI)		NA	NA		NA	NA		NA	NA
	CC[$]	69 (75.0)	66 (73.3)	68 (70.8)										

After Bonferroni's correction the significance at $P < 0.05/8 = 0.00625$. Homozygous = comparing the likelihood of individuals with two copies of the risk allele versus individuals with no copies of the risk allele; heterozygous = comparing the likelihood of individuals with one copy of the risk allele versus individuals with no copies of the risk allele; NA = not available. [$]Homozygous for the risk factor.

replication (Alexopoulou et al., 2001; Liu et al., 2008). After activation by specific ligands such as dsRNA (TLR3-specific), TLR-mediated signaling pathways are activated to induce the expression and secretion of proinflammatory cytokines as well as angiogenic factors (Park et al., 2007; van Beijnum et al., 2008). The investigation of associations between TLR polymorphisms and AMD has been inconsistent. Yang et al. (2008) reported that rs3775291 (L412F) in *TLR3* conferred protection against GA. The minor allele frequencies of rs3775291 were between 21 and 25% in patients with GA and between 31 and 34% in the control groups in the Caucasian sample sets. However, Edwards et al. (2008) could only find the association of rs3775291 with AMD before correcting for multiple testing, and the minor allele frequencies of rs3775291 differed from those in Yang et al. (31.9% in the AMD group and 24.8% in the control group). Edwards et al. did not further stratify the patient groups by AMD subtype. Moreover, Yang et al. did not adjust for age in their analysis, although the average age of the controls differed from that of the patients with GA by more than 5 years. This could have led to a fallacious association because AMD is strongly age-related. Other studies with primarily Caucasian subjects, adjusted for age and gender, did not detect an association between the rs3775291 SNP and either GA or neovascular AMD (Edwards et al., 2008; Allikmets et al., 2009; Cho et al., 2009b) Furthermore, Sng et al. (2011) found that the TLR3 polymorphism rs3775291 was not associated with nAMD or PCV in Chinese. Thus, it remains uncertain if *TLR3* is associated with nAMD or PCV.

The association between PCV and this *TLR3* polymorphism has not been previously investigated. In our age- and gender-adjusted analysis, we provide new data that the *TLR3* gene variant is not associated with either nAMD or PCV in Chinese. This is consistent with Sng et al. (2011) and Zhou et al. (2011), who proposed that the TLR3 mutation reduces binding capacity to dsRNA, protecting against GA rather than the neovascular phenotype. Moreover, genes that have been implicated in the pathogenesis of nAMD and PCV are usually factors likely involved in ischemia, inflammation, and local synthesis and secretion of angiogenic factors (Swaroop et al., 2007; Veritti et al., 2012).

In conclusion, our results indicate that there is no association between the *TLR3* gene variant and either nAMD or PCV in northern Chinese patients, contrary to what has been implicated in Caucasian AMD patients. However, large sample size studies need to be performed to validate this result. Because the incidences of PCV in the Chinese and Japanese populations with nAMD have been high, compared with the much lower incidence observed with Caucasians (Lafaut et al., 2000; Scassellati-Sforzolini et al., 2001; Liu et al.. 2007; Maruko et al., 2007), different populations appear to have different genetic risks, and further investigations into the genetic basis of AMD and PCV in Asians are required.

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