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Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration

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Age-related macular degeneration (AMD) is a genetically heterogeneous disease that leads to progressive and irreversible vision loss among the elderly. Inflammation, oxidative damage, cholesterol metabolism and/or impaired function of retinal pigment epithelium (RPE) have been implicated in AMD pathogenesis. We examined toll-like receptor 4 (TLR4) as a candidate gene for AMD susceptibility because: (i) the *TLR4* gene is located on chromosome 9q32–33, a region exhibiting evidence of linkage to AMD in three independent reports; (ii) the TLR4-D299G variant is associated with reduced risk of atherosclerosis, a chronic inflammatory disease with subendothelial accumulation; (iii) the TLR4 is not only a key mediator of proinflammatory signaling pathways but also linked to regulation of cholesterol efflux and (iv) the TLR4 participates in phagocytosis of photoreceptor outer segments by the RPE. We examined D299G and T399I variants of TLR4 in a sample of 667 unrelated AMD patients and 439 unrelated controls, all of Caucasian ancestry. Multiple logistic regression demonstrated an increased risk of AMD in carriers of the G allele at TLR4 residue 299 (odds ratio = 2.65, $P = 0.025$), but lack of an independent effect by T399I variant. TLR4-D299G showed an additive effect on AMD risk (odds ratio = 4.13, $P = 0.002$) with allelic variants of apolipoprotein E (APOE) and ATP-binding cassette transporter-1 (ABCA1), two genes involved in cholesterol efflux. Interestingly, the effect of TLR4, APOE and ABCA1 variants on AMD susceptibility was opposite to that of association with atherosclerosis risk. Our data provide evidence of a link between multiple diverse mechanisms underlying AMD pathogenesis.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the elderly population of developed countries (1–3). This progressive degenerative disease primarily involves the retina and the retinal pigment epithelium (RPE) in the macular region. As in atherosclerosis and Alzheimer's disease, extracellular deposits of proteins and lipids (called 'drusen') are clinical hallmarks of AMD (4,5). Pathological characteristics of advanced disease include the presence of large macular drusen (LMD), geographic atrophy (GA) and/or choroidal neovascularization (CNV) (6,7).

AMD is believed to result from interactions between multiple genetic variants and environmental factors (8). The strongest identified risk factors are advanced age and family history, though smoking, hypertension and many other risk factors have also been implicated (9,10). Recent studies have begun to establish the importance of genetic variations in the development of AMD. An association between AMD and allelic variants of apolipoprotein E (APOE) has been widely documented; specifically, the APOE-ε4 allele is

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linked to lower risk of disease and the APOE-ε2 allele with higher risk (11–14). In addition, a significant association between AMD and a polymorphism (Y402H) in the complement factor H (CFH) gene on chromosome 1q32 has been identified (15–17). Carriers of the H allele have 4.74-fold increased risk of AMD (S. Zareparsi *et al.*, submitted for publication). CFH is involved in regulation of complement activation, which is an essential component of innate immunity (18). Genome-wide linkage analyses of affected family members have revealed a number of chromosomal regions that may harbor AMD susceptibility genes; several of these are detected by more than one study (19–25). A non-conserved variation Q5345R in fibulin 6 (hemicentin-1) was recently shown to segregate with the disease in a large AMD family originally mapped to chromosome 1q31 (26,27); however, this change does not appear to play a significant role in AMD (22,23,28). Despite the phenotypic similarities with early-onset macular diseases, genes associated with these diseases do not appear to significantly contribute to AMD susceptibility (29–31). However, as a mutation in fibulin 3 (an extracellular matrix protein) underlies drusen formation in a macular disease phenotypically similar to AMD (32,33), a mutation screen of five other fibulin genes was performed in AMD and control individuals. This study detected missense mutations in fibulin 5 in 1.7% of AMD patients (34).

AMD and atherosclerotic cardiovascular disease appear to share common pathogenic mechanisms: both are characterized by lipid deposition (drusen and plaques) and thickening of connective tissue (Bruch's membrane and arterial intima) (35,36). Cardiovascular disease and hypertension have also been reported as risk factors for AMD (reviewed in 10). Genetic variations in genes involved in inflammation, oxidative stress and cholesterol transport have been implicated in susceptibility to atherosclerosis (37) and the pathogenesis of AMD (7,38–40). In addition, elevated levels of C-reactive protein (CRP) are associated with atherosclerotic cardiovascular disease (reviewed in 41) and AMD (42). In contrast, the effect of APOE alleles on AMD risk is the opposite of their effect on the risk for atherosclerosis (43).

Toll-like receptors (TLRs) are involved in innate immunity and pathogen recognition (44,45). Of these, TLR4 has been implicated in modulating susceptibility to atherosclerosis, with the G allele of the D299G variation being associated with reduced risk (46,47). Notably, the *TLR4* gene is located within the region on chromosome 9q32–33 that has been reported by our group (22) and others (20,23) to harbor a potential AMD susceptibility locus. TLR4 is not only a key mediator of pro-inflammatory signaling pathways, but also linked to regulation of cholesterol efflux (48,49). Specifically, TLR4 stimulation by lipopolysaccharide (LPS) inhibited the expression of APOE and ATP-binding cassette transporter-1 (ABCA1) in macrophages (48). More recently, TLR signaling was shown to mediate multiple steps (e.g. phagosome maturation) during phagocytosis (50), and TLR4 was specifically demonstrated to participate in the phagocytosis of photoreceptor outer segments (POS) by the RPE (51), an important function which, when impaired, may lead to AMD pathology (4). Involvement of TLR4 during RPE phagocytic process was a specific response to human POS and was followed by a wave of metabolic and calcium signaling including release of oxidants (51).

In this report, we examined whether variations in TLR4 are associated with AMD susceptibility and what, if any, modifying effects they may have on the existing association between APOE and AMD. Our findings provide strong evidence in support of the importance of RPE function in POS phagocytosis and the involvement of cholesterol transport and local inflammatory response in the pathogenesis of AMD.

RESULTS

Role of TLR4 genetic variations in AMD susceptibility

We screened a cohort of 667 AMD patients and 439 controls for the TLR4-D299G variation, which has been widely studied for its association with atherosclerosis. Genotypic counts and allelic frequencies for TLR4-D299G are summarized in Table 1. There was no evidence that this variant was not in Hardy–Weinberg equilibrium in either AMD patients ($\chi^2 = 0.56$, $P > 0.25$) or controls ($\chi^2 = 0.94$, $P > 0.25$). The frequency of the G allele was increased in patients when compared with controls (0.06 versus 0.03, $P = 0.001$). Odds ratio (OR) calculations indicated that the carriers of the G allele have at least a 2-fold increased risk of developing AMD (Table 2). The association with the G allele remained significant when age and sex were included as covariates in the logistic regression analysis (Table 2).

We also used maximum likelihood model fitting to dissect the impact of the D299G polymorphism on disease susceptibility. As the allele is quite rare, our results cannot distinguish between unconstrained penetrance models and dominant, recessive, additive and multiplicative models of disease susceptibility. Nevertheless, there is strong evidence for association ($\chi^2 = 12.79$, $P = 0.0017$ for the unconstrained penetrance model; $\chi^2 = 11.89$, $P = 0.0006$ for the dominant model) and our results suggest that the G allele has a population frequency of 0.037 (95% CI: 0.029–0.048) and is associated with a genotype relative risk of 1.80 (95% CI: 1.31–2.41) in heterozygotes and 1.90 (95% CI: 0.33–4.75) in homozygotes.

We screened 11 patients (including two homozygous individuals) and 10 controls, all with the G allele of D299G for additional single nucleotide polymorphisms (SNPs) in the *TLR4* gene by sequencing. The only other variation detected in this sample was the T399I variant. Previous studies had contradictory findings on the co-segregation of these two SNPs [46,47,52]. We therefore screened most of our cohort of AMD patients and controls for the T399I variation and there was no evidence that this variant was not in Hardy–Weinberg equilibrium in either AMD patients ($\chi^2 = 0.99$, $P > 0.25$) or controls ($\chi^2 = 0.58$, $P > 0.25$). We detected an increased frequency of I allele in AMD patients when compared with controls (0.08 versus 0.03, $P = 0.001$) (Table 1). On its own, carriers of the T399I-I allele had a 2.4-fold increased risk of developing AMD (Table 2). However, when both polymorphisms were included in the multiple logistic regression model, only the G allele of D299G was associated with a significant 2.65-fold increased risk of AMD, whereas the I allele of T399I did not reveal a significant association (Table 2). This may reflect the observation that a higher proportion of AMD patients than controls carry the risk allele for D299G

Table 1. Genotypic counts and allele frequencies for the TLR4 variants

	AMD	Controls
TLR4 (D299G) (A1063G)		
DD	584 (0.876)	412 (0.941)
DG	81 (0.121)	25 (0.057)
GG	2 (0.003)	1 (0.002)
D allele	0.94	0.97
G allele	0.06	0.03
TLR4 (T399I) (C1363T)		
TT	513 (0.854)	221 (0.932)
TI	86 (0.143)	16 (0.068)
II	2 (0.003)	0 (0)
T allele	0.92	0.97
I allele	0.08	0.03

Table 2. OR for the two variants of the *TLR4* gene

	OR	*P*-value	95% CI
TLR4-D299G			
Unadjusted	2.25	0.001	1.43–3.56
Age and sex-adjusted	2.42	0.001	1.43–4.08
TLR4-T399I			
Unadjusted	2.37	0.002	1.36–4.13
Age and sex-adjusted	2.37	0.003	1.33–4.22
TLR4-D299G and *TLR4-T399I*			
G allele	2.65	0.025	1.13–6.25
I allele	1.28	0.52	0.61–2.69

Table 3. Carrier frequencies for the two variants of the *TLR4* gene

	AMD	Controls
TLR4		
D299G=D and T399I = T	499 (83%)	218 (92%)
D299G=D and T399I = I	21 (3.5%)	8 (3.4%)
D299G=G and T399I = T	14 (2.3%)	3 (1.3%)
D299G=G and T399I = I	67 (11.2%)	8 (3.4%)

Table 4. OR for the interactive and additive effects of the three susceptibility gene variants

	OR	*P*-value	95% CI
TLR4 and *APOE*			
TLR4 (G+)	3.66	0.05	1.00–13.4
APOE (ε4−)	1.43	0.03	1.05–1.95
Interaction	0.57	0.42	0.14–2.28
TLR4, APOE and *ABCA1*			
TLR4 (G+)	2.10	0.01	1.17–3.78
APOE (ε4−)	1.40	0.03	1.03–1.89
ABCA1 (K+)	1.00	0.97	0.78–1.29
Interaction	1.31	0.61	0.47–3.65
TLR4 (G+) and *APOE (ε4−)*			
Additive	2.97	0.0001	1.69–5.20
TLR4 (G+) and *ABCA1 (K+)*			
Additive	2.64	0.008	1.29–5.42
TLR4 (G+), ABCA1 (K+) and *APOE (ε4−)*			
Additive	4.13	0.002	1.67–10.2

but not T399I. Similar frequencies of carriers of the risk allele for T399I but not D299G were observed in both patients and controls (Table 3). The significant association in our cohort therefore appears to be with the TLR4-D299G variant.

We then examined whether the association of TLR4-D299G correlated with a specific AMD subtype. The frequency of the G allele was slightly higher in patients with GA or LMD when compared with patients with CNV (0.08 versus 0.06); however, all three groups had higher frequency of the G allele relative to controls. The frequency of the G allele was higher in both familial (0.06) and sporadic (0.06) AMD patients when compared with controls.

Interaction of TLR4-D299G with ABCA1-R219K and APOE variants

Given that ABCA1 and APOE are downstream in TLR4 signaling pathway (48,49), participate in cholesterol metabolism and are associated with atherosclerosis (43,53), we determined whether ABCA1 and APOE variants interact with TLR4-D299G in contributing to AMD susceptibility. The frequency of the K allele of ABCA1-R219K (associated with reduced risk of atherosclerosis) (53) was similar between AMD patients and controls (0.29 versus 0.29, $P > 0.5$). Consistent with previous studies, APOE-ε4 allele frequency was reduced in patients when compared with controls (0.10 versus 0.14, $P = 0.02$) and ε2 allele frequency was slightly higher in patients than controls (0.09 versus 0.07, $P > 0.2$). We therefore tested for an interaction between TLR4 and APOE susceptibility variants associated with the risk of developing AMD. No significant interaction was observed between the APOE-ε4 and the G allele of TLR4-D299G (Table 4). We then examined whether there was an additive effect of the risk alleles for both genes. The presence of the TLR4-D299G-G allele and the absence of the APOE-ε4 allele were associated with a 3-fold increased risk of AMD (Table 4). We also evaluated an interactive or additive effect of TLR4-D299G with ABCA1, as it is regulated by TLR4 signaling. There was no significant interaction between TLR4-D299G, ABCA1-R219K and APOE on AMD susceptibility (Table 4). Nevertheless, ABCA1-R219K further contributed to the additive effect and the risk of AMD was increased by 4-fold in carriers of the risk alleles for all three genes (G+, K+ and ε4−) when compared with those without the risk alleles (G−, K− and ε4+) (Table 4).

It has been reported that the AMD patients with the APOE-ε2ε3 genotype had an earlier age at diagnosis of AMD when compared with AMD patients with the APOE-ε3ε3 genotype (14). In our cohort of AMD patients, average age at diagnosis of AMD was similar among ε2ε3 patients (71.2 ± 8.4), ε3ε3 patients (71.8 ± 8.9) and ε3ε4 and ε4ε4 patients (69.7 ± 8.7). Age at diagnosis of AMD did not vary significantly in the presence of the TLR4-D299G-G allele (70.6 ± 9.3 versus 71.4 ± 8.7) or the ABCA1-R219K-K allele (71.7 ± 8.7 versus 70.9 ± 8.8). Moreover, age at diagnosis of AMD was similar between patients with risk alleles for all three genes and patients without the risk alleles (72.2 ± 9.7 versus 70.0 ± 8.5).

DISCUSSION

Extensive studies suggest that compromised RPE function, local inflammation, oxidative damage and altered cholesterol/lipid metabolism contribute to AMD pathogenesis (4,5,36,39,54,55). Molecular mechanisms that link these diverse physiological changes to AMD are poorly understood. In this report, we present novel findings of an association between the D299G variant of the *TLR4* gene and the susceptibility to AMD. Another variant T399I does not appear to be independently associated. Additional investigations are warranted to determine whether the association with increased risk of AMD is with D299G or possibly with an allele in linkage disequilibrium. Both variations, D299G and T399I, are present in the extracellular domain of TLR4 and result in differential response to TLR4 ligand, LPS (56); hence, altered TLR4 function may contribute to AMD pathology.

A quick evaluation of genetic data had revealed a possible AMD susceptibility locus at 9q32–33 in three independent studies (20,22,23). This chromosomal region includes TLR4, a member of the toll-like receptor family of proteins which confer viral or bacterial recognition and stimulate proinflammatory pathways leading to anti-pathogen response (57). TLR4 has been linked to atherosclerosis risk and shown to participate in signaling crosstalk with proteins involved in cholesterol metabolism (46,48). TLR4 was also recently shown to be involved in phagocytosis of POS by the RPE and participate in subsequent transmembrane signaling events (51). Our data suggest that altered TLR4 signaling by D299G variant may influence phagocytic function of RPE, which in turn may contribute to RPE damage.

Another significant finding, reported here, is the additive effect of TLR4-D299G with variations in APOE and ABCA1. The link between the three genes is supported by a recent study that induction of TLR4 leads to reduced expression of several genes (including ABCA1 and APOE) activated by liver x receptors (LXRs) (48), which in turn are stimulated by physiological concentrations of oxidized derivatives of cholesterol (58). TLR4 can also directly suppress ABCA1 promoter activity. These genes play an important role in atherosclerosis etiology, as APOE-deficient mice that also lacked TLR4 had reduced atherosclerosis, reduced plaque lipid content, reduced circulating levels of proinflammatory cytokines including monocyte chemotactic protein-1 (MCP1) and decreased macrophage recruitment (59). Interestingly, mice that lack MCP1 or its receptor have been reported to develop AMD-like symptoms at old age (55). Scavenging of oxidized lipids/cholesterol and their subsequent efflux is beneficial, but an overwhelming lipid load may activate an inflammatory response (60), thus LXR-regulated genes may play an important additional role in AMD.

Our study highlights an interesting relationship between AMD and atherosclerosis. Here, we show that the variants reported to correlate with reduced risk of atherogenesis (TLR4-D299G, ABCA1-R219K and APOE-ε2/3) are associated with increased risk of AMD. A few reports have suggested cardiovascular disease and hypertension as risk factors for AMD (reviewed in 10). Esterified and unesterified cholesterol have been observed to accumulate with age in human Bruch's membrane, which separates the RPE and the choriocapillaris similar to the intima of large, atherosclerosis-prone arteries (36). The finding of an association of AMD susceptibility with variants in the three genes (TLR4, APOE and ABCA1) implicates a role for innate immunity and cholesterol transport in the AMD progression. It will be of interest to directly investigate the relationship between AMD and atherosclerosis in combination with genetic variations of TLR4, APOE and ABCA1.

The newly reported association with Y402H variation in CFH does not affect the association between AMD and TLR-D299G. In both carriers and non-carriers of the H allele, those with the G allele have increased risk of AMD (data not shown). An important criterion for acceptance of an association is independent validation (61). Additional studies are therefore necessary to replicate our findings. As indicated by power calculations, such studies should consist of a large sample size ($N \geq 420$ cases and controls) to detect the 2-fold increased risk with sufficient statistical power (at least 80% at $P = 0.05$) given the frequency of the G allele in our cohort.

In conclusion, we report a novel association between the D299G variant in the *TLR4* gene and the AMD susceptibility and provide genetic evidence and possible link in support of the involvement of impaired RPE function, innate immunity and cholesterol metabolism in AMD pathogenesis.

MATERIALS AND METHODS

Subjects

This study consisted of 667 unrelated patients with AMD and 439 unrelated controls, all of Caucasian ancestry. The majority of the participants were recruited from the clinics of the Kellogg Eye Center at University of Michigan and resided in the same geographical location (the state of Michigan). The recruitment and characterization protocols were described previously (13,22). Briefly, fundus findings in each eye were classified based on a standardized set of diagnostic criteria established by the International ARM Epidemiological Study (6). Affected individuals with CNV ($N = 401$) consisted of those with CNV in both eyes and/or CNV in one eye and GA/LMD in the other eye. Patients with GA ($N = 159$) included those with GA in both eyes and/or GA in one eye and LMD in the other eye. Patients with LMD ($N = 107$) had bilateral findings of LMD. Of the control participants, 277 were collected as part of the AMD study; the individuals were considered unaffected if they were over the age of 68 at their last ophthalmic examination, did not have a family history of AMD in more than one family member and did not have any AMD findings in either eye. Controls were on the average ~4 years younger than AMD patients (75.1 ± 5.0 versus 79.0 ± 7.9). However, the controls were ~3.8 years older than the average diagnosis age of patients (71.3 ± 8.7) and still free of any signs of AMD in either eye. An additional 162 controls recruited as normal controls for the glaucoma genetics study at the Kellogg Eye Center were included; review of available records in these individuals did not indicate presence of clinical phenotype of AMD or glaucoma. These controls were younger (68.2 ± 10.8) but had similar allele frequencies for all three genes examined as the controls recruited by the AMD study. This research

was approved by the Institutional Review Board at the University of Michigan and adhered to the tenets of the Declaration of Helsinki. All subjects signed an informed consent.

Genotyping and sequence analysis

Genomic DNA was extracted from peripheral blood leukocytes. Genotyping for the TLR4-D299G (rs4986790) and T399I (rs4986791) polymorphisms were performed as described by allele-specific PCR and restriction digestion (62). To screen for additional SNPs reported for the largest exon for *TLR4* shared by all isoforms (63), the 2876 bp of coding region was sequenced with a set of five overlapping primers designed using primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): fragment 1—forward 5′-ATGGGGCATATCAGAGCCTA-3′ and reverse 5′-TTGTTCTAAGCCCAAGAAGTTTG-3′; fragment 2—forward 5′-TGGCTTGAGTTTCAAAGGTTG-3′ and reverse 5′-AAGGCAGAGCTGAAATGGAG-3′; fragment 3—forward 5′-CGGTCCTCAGTGTGCTTGTA-3′ and reverse 5′-CTGTCCTCCCACTCCAGGTA-3′; fragment 4—forward 5′-TGAGCAGTCGTGCTGGTATC-3′ and reverse 5′-GGGTTCCTTGACTGAGTTGG-3′ and fragment 5—forward 5′-AAGGAGCTTCCAGTGCAGAG-3′ and reverse 5′-GTCACCCCGGGTTTATCAG-3′. A 700 bp region in the promoter of *TLR4* reported to contain two SNPs (52) was also sequenced using forward primer 5′-GATGGAGTCTACAAGAGTTTGTGC-3′ and reverse primer 5′-TTTGGATACGTACTCAGAAGTGAGA-3′. Sequencing was performed by the University of Michigan DNA sequencing core facility. APOE and ABCA1-R219K genotypes were obtained using previously described methods by PCR and restriction digestion (53,64).

Statistical analysis

Allele frequencies were estimated by allele counting. Chi-square analyses of Hardy–Weinberg equilibrium for genotypes were performed for patients and controls. The results were also tested using a recently described exact test (65). Allele frequency differences were tested using Fisher's exact test for 2×2 contingency tables and empirical *P*-values are reported. We evaluated the evidence for different genetic models by maximum likelihood, fitting one allele frequency and three prevalence parameters. We constrained the overall disease prevalence to 20%, which is appropriate for this age group. Unconstrained maximization produced similar results. Logistic regression was used to calculate OR, 95% CI, *P*-values and to adjust for covariate effects by age and sex. Effect of the two TLR4 variations on AMD risk was examined by multiple logistic regression under a main effect model. The estimated OR and corresponding *P*-value under multiple logistic regression are analogous to the type III sums of square analysis under a general linear model (66). Additive effects between the TLR4-D299G and/or APOE and/or ABCA1-R219K were analyzed by logistic regression, where those carrying the risk alleles for any given combination of SNPs were compared with those who lacked the risk alleles for those SNPs (e.g. TLR4-G and ABCA1-K carriers compared with TLR4-DD and TLR4-RR individuals). All analyses were performed using the SPSS Software (Release 11.0).

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