Analysis of Rare Variants in the Complement Component 2 (*C2*) and Factor B (*BF*) Genes Refine Association for Age-Related Macular Degeneration (AMD)

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Purpose. Several single-nucleotide polymorphisms (SNPs) in the *C2* and *BF* genes have been associated with age-related macular degeneration (AMD) in Caucasian populations from the United States. The study was conducted to evaluate whether these SNPs are also associated with AMD in persons of Anglo-Celtic ethnicity in an Australian population.

Methods. Included in the study were 565 persons with AMD and 204 ethnically matched control subjects. All participants completed a standard health questionnaire, were given a fundus examination, and provided a blood sample for DNA extraction. Alleles were determined by a matrix-assisted desorption ionization–time of flight (MALDI-TOF)–based approach followed by statistical analysis.

Results. The *C2* and *BF* genes indicated significant association with AMD of only two SNPs; rs547154 (IVS10) in the *C2* gene ($P = 9.1 \times 10^{-5}$) and rs641153 (R32Q) in the *BF* gene ($P = 7.0 \times 10^{-5}$). No association with AMD was found for SNP rs9332739 (E318D) in the *C2* gene or for rs4151667 (L9H), rs1048709 (R150R), rs4151659 (K565E), or rs2072633 (IVS17) in the *BF* gene. A protective haplotype of variants IVS10 and R32Q was associated with AMD (OR 0.29, 95% CI 0.20–0.42).

Conclusions. In this study, the association of the IVS10 and R32Q variants in the *C2* and *BF* genes in AMD was replicated. Haplotype analysis indicated association of these variants with AMD in an Australian population. Both IVS10 and R32Q variants were in strong linkage disequilibrium with each other ($r^2 = 0.96$). Although the E318D and L9H variants have shown association with AMD in previous studies, the findings were not in agreement. This demonstrates a refined pattern of association of these rare variants with AMD. (*Invest Ophthalmol Vis Sci.* 2009;50:540–543) DOI:10.1167/iovs.08-2423

Age-related macular degeneration (AMD) (ARMD1; MIM 603075) is the leading cause of uncorrectable vision loss and blindness in developed countries, and in Australia it contributes to up to 50% of all blindness (presenting visual acuity < 6/60).[1] A genetic predisposition to this disease has clearly been shown with several genes having now been identified, of which several are inflammatory genes, including the complement factor H gene and the *C3* gene of the alternative pathway.[2–6] Additional support for the involvement of an inflammatory involvement in AMD comes from the association of the two paralogous genes complement component 2 (*C2*; MIM 217000) and factor B (*BF*; MIM 138470), located within the gene-rich class III region of the HLA complex on chromosome 6.[7–9] Moreover, *BF* and *C2* are involved in the initiation of the alternative complement cascade and the activation of the classic component pathway, respectively.

In three previous studies based on American case control cohorts, with one including a family study,[9] the association of the *C2* and *BF* genes with AMD has been examined.[7–9] In two of these studies, the AMD subtypes of early geographic atrophy (GA) and choroidal neovascular (CNV) AMD were investigated.[7,9] The third study was performed to investigate only late-stage AMD.[8] These reports consistently showed involvement of several single-nucleotide polymorphisms (SNPs), including E318D and IVS10 of the *C2* gene and L9H and R32Q of the *BF* gene, with AMD. Each study held the consensus view that each of these SNPs is involved in protection against AMD.

The purpose of our study was to undertake a replication of work on previously identified SNPs in the *C2* and *BF* genes in an Australian AMD case–control cohort, to investigate the SNPs' involvement in AMD.

Methods

Subjects

All individuals in this study were a part of our AMD Inheritance Study (AMDIS). All individuals with AMD were identified through either outpatient clinics at the Royal Victorian Eye and Ear Hospital (RVEEH) or private ophthalmology practices in Melbourne and had an Anglo-Celtic ethnic background. Individuals with AMD were included if they had drusen > 125 μm. Control subjects were obtained from the same community as part of a large population-based epidemiologic eye study, the Melbourne Visual Impairment Project (VIP) or through aged-care nursing homes. Control individuals were included if they presented with a normal fundus (<10 hard drusen <63 μm in size) and no altered macular pigmentation. At the time of ascertainment a clinical examination was performed, a fundus photograph obtained, and a blood sample collected for DNA analysis. In addition, all participants completed a standard risk factor and disease history questionnaire. Smoking was assessed by asking whether individuals were current or past smokers or had never smoked. All participants in our study were unrelated.

Written informed consent was obtained from all individuals, and ethics approval for the project was provided by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne. The study was conducted in accordance with the Declaration of Helsinki and according to the National Health and Medical Research Council of Australia's statement on ethical conduct in research involving humans, revised in 1999.

Genotyping

We chose to genotype SNPs that had been previously investigated,[7] and included rs9332739 (E318D) and rs547154 (IVS10) in the *C2*

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TABLE 1. Primer Details for Genotyping of SNPs from the *C2* and *BF* Genes

SNP ID	PCR 1st Primer	PCR 2nd Primer	Amplicon Length (bp)	Extension Sequence Primer
rs9332739	ACGTTGGATGTGTCTGTCCTGAACGACAAC	ACGTTGGATGCCCGTACCTTTATAGTTGGC	99	GCATTTTCCAGGCTGCTGATCAC
rs547154	ACGTTGGATGAGTGAGCTTTGCCCTCCTTG	ACGTTGGATGAAGTGAGGGGCACTGTGTC	109	GGGGCACTGTGTCCAGGTTCCCAA
rs4151667	ACGTTGGATGCAAGAGGCCCAAGATAAAGG	ACGTTGGATGCTTCTCTCCTGCCTTCCAAC	100	CAATCTCAGCCCCCAAC
rs641153	ACGTTGGATGCCTTTCTCTTCAGGTGTGAC	ACGTTGGATGTTGATCTCTACCCCCTCCAG	103	CAGAGAGCAGGATCCCTGGGGC
rs1048709	ACGTTGGATGCGTTGTCACAGATCGCTGTC	ACGTTGGATGTCTTTCCACTGCTATGACGG	120	TGCCAAGTGAATGGCCG
rs4151659	ACGTTGGATGGAAGTAGTCCTATTTCACCC	ACGTTGGATGATCAGGGCAACGTCATAGTC	109	TTCAGGAATTCCTGCTTCTT
rs2072633	ACGTTGGATGTCAAGAACGAGGCTGAGCTG	ACGTTGGATGTAATCCTGGAAGCATGGCTG	106	AAGCATGGCTGTTCCTGCTTG

gene, and rs4151667 (L9H), rs641153 (R32Q), rs1048709 (R150R), rs4151659 (K565E), and rs2072633 (IVS17) in the *BF* gene.

Genotyping of all SNPs was performed as previously described[10] using a genetic analysis system (MassARRAY platform; Sequenom, San Diego, CA) through the Australian Genome Research Facility (AGRF; Brisbane, Australia). The polymerase chain reaction (PCR) primers and primers (MassExtend; Sequenom) used in our study are listed in Table 1.

Statistical Analysis

Study characteristics including sex and age at ascertainment or diagnosis of the participants with and without AMD were compared by using the χ^2 test or independent sample *t*-test. Hardy-Weinberg equilibrium (HWE) was undertaken to assess whether genotypes fell within a standard distribution with the software program JLIN: a Java-based linkage disequilibrium (LD) plotter.[11] Deviations of genotype frequencies in controls and cases from those expected under Hardy Weinberg were assessed by χ^2 tests. Allelic associations with AMD were investigated with the UNPHASED software,[12] and results were presented as odds ratio (OR; 95% [confidence interval] CI). Genotype frequencies were compared between total AMD cases and controls and also between AMD subtypes and controls by χ^2. We applied the additive model as the best-fit model to determine genotype distributions. Haplotype analysis was performed by using the same UNPHASED software.[12] Participants' characteristics and genotype associations were performed with commercial software (SPSS; ver. 14.0; SPSS Inc., Chicago, IL).

RESULTS

A total of 769 individuals were included in this study. Of these, 565 (73%) individuals presented with AMD with a mean age of diagnosis of 73.4 years. In addition, 204 (27%) unaffected individuals with a similar mean age of 72.4 years were also recruited ($P = 0.1$; Table 2). There were significantly more females with AMD than there were males, and a higher percentage of females overall compared with males ($P < 0.02$; Table 2).

There was no evidence of departure from HWE for any of the SNPs analyzed in our study ($P > 0.05$; data not shown). Allelic association, after adjustment for smoking, was evident for AMD for the variant IVS10 of the *C2* gene, with an OR of 2.28 (95% CI, 1.52–3.40) and for the variant R32Q of the BF gene with an OR of 2.31 (95% CI, 1.55–3.45; Table 3). We did not detect any significant allelic association for the variant E318D in the *C2* gene, nor for the variants L9H, R150R, K565E, and IVS17 in the *BF* gene (Table 3).

The genotype distribution in total AMD and control for all SNPs was compared by using χ^2 statistics. In genotype associations, only IVS10 and R32Q were significantly associated with AMD, as was observed in allelic associations (Table 3). After Bonferroni correction for multiple testing, both IVS10 and R32Q variants remained significant in AMD cases compared with controls ($P \leq 0.001$; Table 3).

AMD subtype association analysis was not undertaken, as none of the subtypes was significantly different than the total AMD group in their observed genotype frequencies.

Haplotype analysis revealed only one haplotype (haplotype 2) as being significantly associated with protection against AMD with an odds ratio of 0.29 (Table 4). No other haplotype combinations were found to be significant in our analysis (Table 4).

DISCUSSION

This is the first non-U.S. Caucasian study in which the association of SNP variants in the *C2* and *BF* genes in AMD was examined. In the present study we confirmed previous significant findings for the two SNPs, IVS10 in the *C2* gene and R32Q in the *BF* gene, in association with AMD.

The IVS10 and R32Q variants appear to be in complete LD ($r^2 = 0.96$) in our AMD cohort (Fig. 1). These findings agree with previous studies that showed these two variants to be in strong LD.[7–9] However, R32Q was not in LD with any of the other SNPs tested in this study. Previous studies also implicated the E318D and L9H variants in the *C2* and *BF* genes, respectively, as being associated with AMD as well as being in high LD with each other.[7–9] Although we did find them to be in high LD ($r^2 = 0.97$), our findings do not support their association with AMD. A previous report also indicated no significant association of the E318D and L9H variants in the family arm of their study or when smoking was included as a covariant in their case–control model.[9] Overall, these findings suggest that the protective effect of the *C2* and *BF* genes resides within the LD block identified by the IVS10 and R32Q variants.

The protective alleles (T at IVS10 and A at R32Q) present as uncommon variants in our study with a frequency of 0.117 and

TABLE 2. Characteristics of AMD, Its Clinical Subtypes, and Controls

Characteristics	Control (%)	Any AMD (%)	*P**	Early (%)	*P**	CNV (%)	*P**	GA (%)	*P**
Total ($N = 769$)	204 (26)	565 (73)		43 (7.6)		421 (74.5)		101 (17.8)	
Male, *n* (%)	84 (41.2)	183 (32.4)	0.02†	8 (18.6)	0.005†	135 (32.1)	0.03†	40 (39.6)	0.79
Female, *n* (%)	120 (58.8)	382 (67.6)		35 (81.4)		286 (67.9)		61 (60.4)	
Age, mean (SD)	72.4 (6.2)	73.4 (7.8)	0.10	71.9 (5.7)	0.65	73.8 (7.7)	0.03†	72.4 (8.7)	0.99

* *P* based on χ^2 (categorical) or independent-sample *t*-test.
† Statistically significant.

TABLE 3. SNP Identity, Location, Nucleotide Position and Allele Association with AMD for the *C2* and *BF* Genes

SNP Information			Genotype Information and Allele Associations with AMD				
				Allele/Genotype Frequency		Allele Association	
SNP Name	SNP Position	Nucleotide Position*	Allele/Genotype	Case	Control	*P*†	OR (95% CI)‡
C2 gene							
s9332739	E318D	32,011,783	C allele	23 (2.2)	11 (3.5)	0.24	1.00
			G allele	1011 (97.8)	303 (96.5)		1.50 (0.76–2.94)
			CC	0 (0.0)	0 (0.0)	0.20	
			CG	23 (4.4)	11 (7.0)		
			GG	494 (95.6)	146 (93.0)		
rs547154	IVS10	32,018,917	T allele	58 (5.5)	47 (11.7)	9.1×10^{-5}	1.00
			G allele	992 (94.5)	353 (88.3)		2.28 (1.52–3.40)
			TT	2 (0.4)	3 (1.5)	<0.001	
			GT	54 (10.2)	41 (20.5)		
			GG	469 (89.5)	156 (78.0)		
BF gene							
rs4151667	L9H	32,022,003	A allele	23 (2.2)	12 (3.7)	0.17	1.00
			T allele	1017 (9897.8)	324 (96.3)		1.59 (0.82–3.09)
			AA	0 (0.0)	0 (0.0)	0.19	
			AT	23 (4.4)	12 (7.4)		
			TT	497 (95.6)	150 (92.6)		
rs641153	R32Q	32,022,159	A allele	58 (5.5)	47 (11.8)	7.0×10^{-5}	1.00
			G allele	1000 (94.5)	351 (88.2)		2.31 (1.55–3.45)
			AA	2 (0.4)	3 (1.5)	<0.001	
			GA	54 (10.2)	41 (20.6)		
			GG	473 (89.4)	155 (77.9)		
rs1048709	R150R	32,022,914	A allele	215 (20.2)	64 (20.4)	0.64	1.00
			G allele	847 (79.8)	250 (79.6)		1.07 (0.80–1.43)
			AA	19 (3.6)	4 (2.5)	0.74	
			GA	177 (33.3)	56 (35.7)		
			GG	335 (63.1)	97 (61.8)		
rs4151659	K565E	32,026,443	A allele	1005 (98.3)	312 (99.4)	0.35	1.00
			G allele	17 (1.7)	2 (0.06)		1.64 (0.54–4.93)
			AA	494 (96.7)	155 (98.7)	0.14	
			GA	17 (3.3)	2 (1.3)		
			GG	0 (0.0)	0 (0.0)		
rs2072633	IVS17	32,027,557	A allele	478 (45.9)	135 (43.0)	0.37	1.00
			G allele	564 (54.1)	179 (57.0)		1.12 (0.87–1.44)
			AA	117 (22.5)	33 (20.9)	0.27	
			GA	244 (46.8)	69 (43.3)		
			GG	160 (30.7)	55 (34.8)		

* Taken from National Center for Biotechnology Information (NCBI; Bethesda, MD) build 36.

† *P* for global associations based on log likelihood ratio statistics for allelic associations and trend based on χ^2 statistics for genotype associations.

‡ OR (95% CI) for allelic associations adjusted for smoking status. ORs are not constructed for genotypes due to rare or no frequencies of wild-type genotypes.

0.118, respectively, in our control subjects. This was similar to the MAF reported in HapMap of 0.109 for IVS10 and 0.117 for R32Q (HapMap). However, these frequencies were approximately double those of 0.055 (IVS10) and 0.054 (R32Q) in our cases. This doubling in observed minor allele frequency (MAF) for IVS10 and R32Q between controls and cases has been reported in prior studies.[7–9] As a consequence, we were only able to detect a TT genotype for IVS10 or an AA genotype for R32Q in 3/204 (1.5%) of controls and 2/565 (0.3%) cases of AMD.

TABLE 4. Haplotype Analysis of Six SNPs in the *C2* and *BF* Genes in the Total AMD Group

Haplotype	rs332739	rs547154	rs4151667	rs641153	rs1048709	rs2072633	Case Frequency	Control Frequency	OR	95% CI	*P*
1	G	G	T	G	G	A	0.21	0.14	1.00	N/A	0.0017
2	G	**T**	T	**A**	G	A	0.05	0.12	0.29	0.20–0.42	0.0052
3	G	G	T	G	A	G	0.005	0.008	0.39	0.08–1.8	0.4362
4	G	G	T	G	A	A	0.197	0.199	0.67	0.45–0.99	0.9075
5	G	G	T	G	G	G	0.512	0.494	0.71	0.49–0.99	0.5298
6	C	G	A	G	G	G	0.024	0.035	0.47	0.32–0.67	0.5098

rs4151659 was not included in the haplotype analysis, as the minor allele frequency of G was less than 2% in both cases and controls, and its inclusion did not allow haplotypes to be determined. Bold letters denote a change in genotype haplotype 2.

* χ^2 (categorical) or independent sample *t*-test.

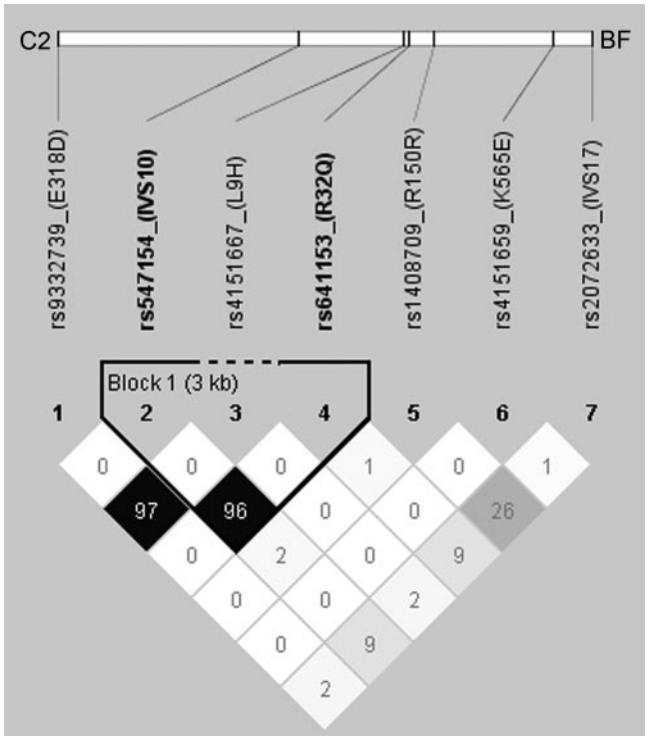

FIGURE 1. Haploview pair-wise LD diagram showing the haplotype blocks across the *C2* and *BF* genes and the relative position of seven SNPs. The numbers inside the *diamonds* refer to r^2 values and show the amount of LD between two markers. *Black*, *gray*, and *white diamonds*: indicate high, medium, and low levels of LD, respectively. The position of each SNP is indicated in brackets. *Vertical line* on the *white bar:* location of the SNP in relation to the gene.

The level of association in the IVS10 and R32Q variants is highlighted by our haplotype analysis. Haplotype 2 showed the protective effect of the changes at both these variants. This finding is in agreement with the original paper by Gold et al.,[7] outlining the association of the *C2* and *BF* genes with AMD.

One of the main challenges in identifying genetic variants in complex diseases using genome-wide association studies (GWAS) is to identify not only common but also rare variants that have small but important roles in disease etiology.[13] The evidence presented herein suggests that the variants in the *C2/BF* genes associated with AMD clearly fall into this rare variant category. It is, therefore, likely that other rare genetic variants exist that also play a role in AMD, and these are as yet to be identified either through linkage or GWAS.

In summary, our data further refine the causative variant for AMD to the LD block identified by either IVS10 in the *C2* gene or the R32Q in the *BF* gene. This observation, along with previous reports that have narrowed the variants to be either E318D or IVS10 in the *C2* gene and L9H or R32Q in the *BF* gene, indicates that IVS10 and R32Q have remained candidate variants associated with AMD throughout all the studies conducted to date. However, our inability to replicate the E318D- or L9H-associated variants identified in the U.S. studies indicates one of the key challenges faced by the Human Variome Project[14] in establishing the role of rare variants in what appear to be similar but subtly different in Caucasian populations in the United States and Australia.

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