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The Involvement of Complement Factor B and Complement Component C2 in an Indian Cohort with Age-Related Macular Degeneration

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Purpose. Genes involved in the complement cascade such as complement factor B (*CFB*) and complement component *C2* have been implicated in age-related macular degeneration (AMD) worldwide. In continuation of the analysis of *CFH* and *LOC387715/HTRA1*, this study was conducted to gain understanding of the role of *CFB* and *C2* in an Indian AMD cohort.

Methods. Single nucleotide polymorphisms in *CFB* and *C2* were screened in a cohort of clinically well-characterized patients with AMD (n = 177) and unaffected normal control subjects (n = 175). Screening was accomplished by a combination of customized genotyping followed by validation through resequencing. In addition, genotyping of two *CFB* variants (rs12614 and rs641153) that were in close proximity had to be resolved by resequencing. Estimates of allele and genotype frequencies, odds ratios, Hardy-Weinberg equilibrium, linkage disequilibrium (LD), and haplotype frequencies were also performed.

Results. Three SNPs in *C2* (rs547154 [IVS10]; $P = 5.4 \times 10^{-11}$) and *CFB* (rs641153 [R32Q], $P = 2.2 \times 10^{-7}$ and rs2072633 [IVS17]; $P = 2.0 \times 10^{-4}$) were strongly associated with reduced risk of AMD. The rs547154 and rs641153 were in strong LD ($D' = 0.90$, 95% CI = 0.81–0.96) and a protective haplotype T-A was observed (OR = 0.10, 95% CI = 0.05–0.20). LD was moderate ($D' = 0.77$, 95% CI = 0.67–0.85) between the rs547154 and the rs2072633 SNPs, and the haplotype T-T generated with these SNPs was relatively less protective (OR = 0.28, 95% CI = 0.18–0.44).

Conclusions. The results of the present study provide an independent validation of the association of rs547154 (*C2*) and rs641153 (*CFB*) SNPs with reduced risk of AMD in an Indian cohort. (*Invest Ophthalmol Vis Sci.* 2010;51:59–63) DOI: 10.1167/iovs.09-4135

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Supported by Fast Track Research Grant DST SR/FT/L-66/2006, Department of Science and Technology, Government of India (IK). SK is the recipient of a senior research fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India.

Submitted for publication June 12, 2009; revised August 1, 2009; accepted August 6, 2009.

Disclosure: **I. Kaur**, None; **S. Katta**, None; **R.K. Reddy**, None; **R. Narayanan**, None; **A. Mathai**, None; **A.B. Majji**, None; **S. Chakrabarti**, None

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Age-related macular degeneration (AMD; OMIM 610149; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) is the third leading cause of blindness among the elderly worldwide and occurs due to the progressive loss of central vision.[1,2] AMD has a relatively higher prevalence in the developed countries[3,4] and has gradually become a major health concern in the developing countries including India due to rapidly changing demographics, life styles, and senescence.[5,6] It is a complex disorder with multifactorial etiology, and genetic susceptibility is a major risk factor.[7,8] Several chromosomal loci have been mapped in AMD, and two major loci on chromosomes 1q32 and 10q26 harboring the complement factor H (*CFH*, OMIM +1343370) and a hypothetical gene *LOC387715* (OMIM +611313), now known as *ARMS2* and a flanking gene *HTRA1* (OMIM 602194) have been characterized.[9–18] Globally, several studies have replicated the association of variants within these genes in AMD across multiple populations.[8,18]

After the discovery of *CFH*, a series of other genes involved in AMD pathogenesis were discovered in the complement cascade.[8] These included two paralogous genes: complement factor B (*CFB*; OMIM 138470) and complement component 2 (*C2*; OMIM +217000) located in the major histocompatibility complex (MHC) class III region on 6p21.[19] The single nucleotide polymorphisms (SNPs) in *C2* (rs9332739 [E318D] and rs547154 [IVS10]) and *CFB* (rs4151667 [L9H] and rs641153 [R32Q]) were observed to be strongly associated with reduced risk of AMD.[19] A strong linkage disequilibrium (LD) was observed between the rs547154 and rs641153 SNPs in two studies of Caucasian populations from the United States[20] and Australia[21] that convincingly replicated these associations in their AMD cohorts. Selective screening of a few *C2* and *CFB* SNPs in two large Caucasian populations that included both familial and case–control cohorts, indicated that rs547154 (*C2*),[22] and rs9332739 (*C2*),[23] and rs4151667 (*CFB*)[23] SNPs, respectively, conferred protection against AMD. A British study of a cohort with neovascular AMD screened with tag-SNPs derived from the HapMap Project harboring the *C2/CFB* region reported significant associations with the previously associated SNPs in this region.[24] The authors also hypothesized a functional involvement of a potential candidate gene *SKIVL2* in AMD that overlay within the LD block of *C2/CFB*.[24]

Thus, it is evident that the *CFB* and *C2* SNPs have been characterized quite extensively in Caucasian populations worldwide, but not in other ethnic groups. Our earlier studies in an Indian AMD cohort had demonstrated the association of *CFH* and *LOC387715/HTRA1* with a risk profile similar to that in Caucasian populations.[25,26] We now sought to understand the involvement of *C2* and *CFB* in the same cohort and to independently validate the association of these SNPs in AMD.

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TABLE 1. Distribution of Minor Allele Frequencies of the *C2* and *CFB* SNPs

Genes	SNP (dbSNP ID)	Location (Amino Acid Change)	Minor Allele	MAF in Cases (n = 177)	MAF in Controls (n = 175)	P	OR (95% CI)
C2	rs9332739	E318D	C	0.958	0.937	0.223	0.66 (0.34–1.30)
	rs547154	Intron 10 (IVS10)	T	0.085	0.274	5.4×10^{-11}	0.24 (0.16–0.38)
CFB	rs4151667	Exon 1 (L9H)	A	0.040	0.063	0.160	0.61 (0.31–1.22)
	rs12614	Exon 2 (R32W)	T	0.148	0.158	0.753	0.92 (0.59–1.44)
	rs641153	Exon 2 (R32Q)	A	0.077	0.258	2.2×10^{-7}	0.28 (0.17–0.46)
	rs1048709	Exon 3 (R150R)	A	0.186	0.211	0.406	0.85 (0.59–1.24)
	rs4151669	Exon 4 (P168P)	A	0.033	0.063	0.111	0.57 (0.28–1.44)
	rs4151670	Exon 5 (Y224Y)	T	0.003	0.003	0.994	0.99 (0.06–15.87)
	rs4151651	Exon 5 (G252S)	A	0.006	0.003	0.570	1.98 (0.18–21.97)
	rs4560093	Exon 8 (R379R)	T	0.003	0.003	0.994	0.99 (0.06–15.87)
	rs4151659	Exon 13 (K565E)	G	0.003	0.000	—	—
	rs2072633	Intron 17 (IVS17)	T	0.206	0.321	2.0×10^{-4}	0.53 (0.38–0.75)

METHODS

Clinical Characterization of the Cases and Controls

The study conformed to the tenets of the Declaration of Helsinki, and prior approval was obtained from the Institutional Review Board of the L.V. Prasad Eye Institute. A cohort of 512 consecutive subjects included patients with AMD (n = 262) and ethnically matched normal controls (n = 250) drawn from the same geographic region of habitat. Clinical characterization of these cases and controls along with the details of clinical examinations have already been described in our earlier publications in this journal.[25,26] AMD in each subject was independently diagnosed by two retina specialists with previously laid out inclusion and exclusion criteria. Written informed consent was obtained from all the subjects before their enrollment in the study.

Selection of Variants (SNPs) in the Candidate Genes

We chose multiple arrays of SNPs in each gene for genotyping. The selection criteria were based on previous evidence of association of these SNPs with AMD. We then chose additional flanking SNPs to the associated SNPs, to enlarge the genomic region being screened. Based on these criteria, we chose a set of 12 SNPs that included the *CFB* (n = 10) and *C2* (n = 2) genes (Table 1).

Genotyping of SNPs

Customized genotyping was undertaken to initially screen these 12 SNPs (GoldenGate Technology; Illumina, Inc., San Diego, CA) according to the manufacturer's protocol. Genotypes were extracted (Bead Studio software, ver. 3.0; Illumina) through a clustering algorithm. Five independent samples were provided as replicates in each 96-well plate for the sake of validation of the genotypes. Further details are available on request. Among the SNPs in the *CFB* gene, we could accommodate only one of the SNPs harboring codon 32 (i.e., rs12614 [R32W]) in the assay (GoldenGate Assay; Illumina). Hence the other SNP rs641153 (R32Q) at this codon was screened by resequencing with appropriate primers by using dye termination chemistry on an automated DNA sequencer (Big Dye Termination on a 3130xl sequencer; ABI, Foster City, CA) according to the manufacturer's guidelines.

Validation of SNPs

Apart from replication of the samples in the same and different plates in the assay, resequencing was performed to validate these SNPs in a subset of samples (BigDye Chemistry; ABI) on an automated DNA sequencer (3130xl; ABI). To validate the three associated SNPs, further resequencing was done in the remaining cohort as well as in the previously assayed subjects. There was total concordance between the genotype calls obtained (Bead Analysis software; Illumina) and the SNP sequencing data.

Statistical Analysis

Allele and genotype frequencies were estimated by the gene-counting method and their significance was calculated by χ^2 statistics. Odds ratios (ORs) were computed to assess the odds of the associated alleles and genotypes. Hardy-Weinberg equilibrium was calculated and haplotype frequencies were estimated with Haploview software (ver. 4.0) which uses the EM algorithm.[27] Permutation tests were performed to assess the extent of association of individual SNPs and haplotype blocks. LD analysis between the SNPs was analyzed using the LD plot function of the software.

RESULTS

Initially, all the 12 SNPs in *CFB* and *C2* were screened in a cohort of 352 subjects (177 AMD cases and 175 controls) by a

TABLE 2. Distribution of Genotype Frequencies of the Associated *C2* and *CFB* SNPs

Genes	SNP (dbSNP ID) (Major Allele > Minor Allele)	Genotypes	Genotype Counts in Cases	Genotype Counts in Controls	P	OR (95% CI)
C2	rs547154 ($G > T$)	GG	149	90		Reference
		GT	26	74	<0.001	0.21 (0.13–0.36)
		TT	2	11	<0.001	0.11 (0.02–0.51)
CFB	rs641153 ($G > A$)	GG	142	95		Reference
		GA	18	53	<0.001	0.23 (0.12–0.41)
		AA	2	10	0.003	0.13 (0.03–0.62)
	rs2072633 ($C > T$)	CC	109	79		Reference
		TC	63	77	0.012	0.60 (0.38–0.92)
		TT	5	19	<0.001	0.20 (0.07–0.53)

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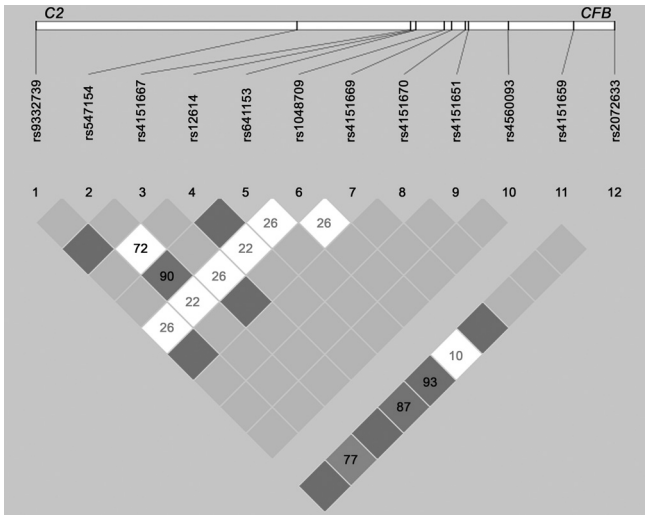

FIGURE 1. An LD map showing the pair-wise LD between the variants (SNPs) in the 6p21 region harboring the *C2* and *CFB* genes in the normal controls in an Indian cohort. The values inside the squares indicate the D′ values of the two SNPs.

genotyping assay (GoldenGate Assay; Illumina). Later, we genotyped only the associated SNPs ($n = 3$) in the remaining cohort ($n = 160$). Since these additional genotypings did not alter the previous results, we have provided only the data of the original cohort for uniformity with the other SNPs. The rs4151659 (K565E) in *CFB* was completely monomorphic in the normal controls and was not included for any further analysis. There was no deviation from Hardy-Weinberg equilibrium among the other SNPs in the normal controls.

Analysis of Variants in the *C2* and *CFB* Genes

Screening of *C2* and *CFB* SNPs revealed one SNP in *C2* (rs547154 [IVS10]) and two SNPs in *CFB* (rs641153 [R32Q] and rs2072633 [IVS17]) that were strongly associated with AMD (Table 1). The normal controls exhibited very high frequencies for the minor alleles of rs547154 ($P = 5.4 \times 10^{-11}$), rs641153 ($P = 2.2 \times 10^{-7}$) and an intron 17 variant rs2072633 ($P = 2.0 \times 10^{-4}$). All the *P* values obtained were significant after Bonferroni corrections for multiple comparisons. The other SNPs in *CFB* did not exhibit any association to AMD in this cohort. The protective alleles of rs547154 and the rs641153 were strongly associated with reduced risk of AMD, as is evident from their ORs (Table 1).

The distribution of genotypes for the three associated SNPs at the *C2* and *CFB* locus in cases and controls are provided in Table 2. Apparently, the presence of the protective allele(s) in the genotype was significantly associated with the controls.

LD and Haplotype Analysis

The extent of LD was assessed between the three associated SNPs in *C2* and *CFB*. A high LD (D′ = 0.90, 95% CI = 0.81–0.96) was observed between the rs547154 (*C2*) and rs641153 (*CFB*) SNPs (Fig. 1). LD was also stronger (D′ = 0.93, 95% CI = 0.83–0.98) between the two *CFB* SNPs rs641153 and rs2072633 but was moderate (D′ = 0.77, 95% CI = 0.67–0.85) between the rs547154 (*C2*) and the rs2072633 (*CFB*) SNPs.

The distribution of estimated haplotype frequencies based on eight SNPs in *C2* and *CFB* are provided in Table 3. The other SNPs were not considered as they had an minor allele frequency (MAF) <3% in the normal population. Only haplotypes with a frequency >5% in the cohort were considered. Haplotypes generated with the three associated SNPs (rs547154, rs641153, and rs2072633) revealed a protective haplotype, T-A-T, that was strongly associated with a reduced risk of AMD ($P = 4.9 \times 10^{-14}$). This association was consistent ($P = 2.5 \times 10^{-14}$), even when the haplotype (T-A) was generated with rs547154 and rs641153 SNPs (Table 3), similar to previous studies.[19,21] Based on permutation tests for individual SNPs and haplotype blocks ($n = 10{,}000$ permutations), we observed that the rs547154 (*C2*) and rs641153 (*CFB*) were the most strongly associated SNPs (permutation $\chi^2 = 43.028$ and 26.818, respectively, $P < 10^{-8}$), and so was the T-A haplotype (permutation $\chi^2 = 58.043$, $P < 10^{-6}$). The rs2072633 SNP had a relatively lesser permutation value ($\chi^2 = 13.461$, $P = 0.003$) that was evident when the rs641153 was replaced by rs2072633 to generate a haplotype with rs547154 (T-T) and the association was relatively weaker (permutation $\chi^2 = 21.547$, $P < 10^{-4}$).

DISCUSSION

The association of variants in *CFB* and *C2* with AMD has been established in multiple Caucasians populations worldwide.[19–24] To the best of our knowledge, this is the first study that unequivocally validates the association of these SNPs in an AMD cohort from India. Genetic associations are more mean-

TABLE 3. Estimated *C2/CFB* Haplotype Frequencies

Haplotypes											
rs9332739	rs547154	rs4151667	rs12614	rs641153	rs1048709	rs4151669	rs2072633	% Cases	% Controls	*P*	OR (95% CI)
C	G	T	C	G	G	G	C	48.7	35.0	2.0×10^{-4}	1.77 (1.30–2.40)
C	**T**	**T**	**C**	**A**	**G**	**G**	**T**	**2.7**	**19.1**	$\mathbf{3.1 \times 10^{-12}}$	**0.12 (0.06–0.24)**
C	G	T	T	G	G	G	C	9.2	8.8	0.865	1.03 (0.61–1.73)
C	G	T	C	G	A	G	C	9.5	8.5	0.658	1.10 (0.66–1.85)
C	G	T	C	G	G	G	T	7.1	5.4	0.364	1.33 (0.72–2.46)
x	G	x	x	G	x	x	C	73.2	61.9	0.001	1.68 (1.22–2.31)
x	**T**	x	x	**A**	x	x	**T**	**2.7**	**21.1**	$\mathbf{4.9 \times 10^{-14}}$	**0.11 (0.06–0.22)**
x	G	x	x	G	x	x	T	12.0	8.7	0.143	1.47 (0.90–2.41)
x	G	x	x	G	x	x	x	85.2	70.4	1.9×10^{-6}	2.43 (1.67–3.53)
x	**T**	x	x	**A**	x	x	x	**2.5**	**20.9**	$\mathbf{2.5 \times 10^{-14}}$	**0.10 (0.05–0.20)**
x	T	x	x	G	x	x	x	6.0	6.5	0.765	0.89 (0.49–1.65)
x	G	x	x	x	x	x	C	78.9	63.1	3.4×10^{-6}	2.21 (1.58–3.09)
x	**T**	x	x	x	x	x	**T**	**8.0**	**23.4**	$\mathbf{2.2 \times 10^{-8}}$	**0.28 (0.18–0.44)**
x	G	x	x	x	x	x	T	12.6	9.5	0.191	1.36 (0.85–2.20)

The bold rows indicate haplotypes based on the combinations of the minor alleles of three strongly associated SNPs in *C2* (rs547154) and *CFB* (rs641153 and rs2072633).

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Table 4. Distribution of Minor Allele Frequencies and ORs of rs547154, rs641153, and rs2072633 across Different Populations Worldwide

Population (Cases, Controls)	rs547154 (*C2*)				rs641153 (*CFB*)				rs2072633 (*CFB*)			
	Freq. Cases	Freq. Controls	*P*	OR	Freq. Cases	Freq. Controls	*P*	OR	Freq. Cases	Freq. Controls	*P*	OR
U.S. (900, 400)[19]	—	—	8.45×10^{-8}	0.44	—	—	6.43×10^{-9}	0.32	—	—	0.044	0.36
U.S. (698, 282)[20]	0.05	0.11	9.2×10^{-6}	—	0.05	0.10	2.3×10^{-5}	—	0.45	0.46	0.592	—
U.S. (187, 168)[22]*	0.025	0.096	0.00011	—	—	—		—	0.33	0.393	0.093	—
U.K. (318, 243)[24]	—	—	—	—	0.05	0.12	0.00008	0.40	0.50	0.44	0.05	1.27
Australia (565, 204)[21]	0.055	0.117	9.1×10^{-5}	—	0.055	0.118	7.0×10^{-5}	—	0.459	0.43	0.37	—
India (177, 175) (Present Study)	0.085	0.274	5.4×10^{-11}	0.24	0.077	0.232	2.2×10^{-7}	0.28	0.206	0.329	2.0×10^{-4}	0.53

* Includes case–control data only.

ingful when they are replicated across multiple ethnic groups from different geographic regions.[28,29] These associations help in fine mapping disease associations and in determining whether effect sizes of the associated alleles are similar or different across populations. Nonreplications in association studies could be attributed to genetic heterogeneity or poor design, but a well-designed case–control study with adequate power provides an opportunity to understand the involvement of alleles with modest disease susceptibility.[30,31] In addition, it provides evidence of the underlying biological mechanism in the disease pathway.[32] Our allelic and haplotype data further confirms the universality of this association in a non-Caucasian population sampled from the Eastern world and provides further support to the implication of the *CFB* and *C2* SNPs in AMD pathogenesis.

The frequency of the protective alleles of rs547154 (*C2*), rs641153 and rs2072633 (*CFB*) were higher in the present cohort than in the Caucasian populations (Table 4). As is evident from the table, the extent of the association based on these SNPs in the Indian cohort was similar to that observed in the Caucasian populations. The extent of LD between these two SNPs (Fig. 1) was also similar to that observed in AMD cohorts from the United States[20] and Australia.[21]

On the other hand, we observed a lower frequency of the protective alleles of rs2072633 SNP in our cohort and a relatively stronger association ($P = 2.01 \times 10^{-4}$) compared with other populations (Table 4). This is also reflected in the strong LD ($D' = 0.93$) between these SNPs (Fig. 1) in our cohort. A moderate association was noted for this SNP in the U.S. ($P = 0.044$)[19] and U.K. cohorts ($P = 0.05$).[24]

Haplotype analysis with the associated SNPs in rs547154 (*C2*) and rs641153 (*CFB*) indicated a protective haplotype T-A that was significantly associated in the Indian cohort ($P = 2.6 \times 10^{-14}$), similar to that in the Caucasians.[19,21] The T-A haplotype exhibited a higher frequency in the normal control (21%) than in other populations.[19,21] The extent of protection conferred by the T-A haplotype (rs547154 and rs641153) was relatively stronger (OR = 0.10, 95% CI = 0.05–0.20) than the T-T haplotype based on rs547154 and rs2072633 SNPs (OR = 0.28, 95% CI = 0.18–0.44).

The rs9332739 (E318D; *C2*) and the rs4151667 (L9H; *CFB*) SNPs did not exhibit any association (Table 1), despite a strong LD between these SNPs in the present cohort (Fig. 1). This finding is in contrast to those in some populations[19,23,24] in which strong association of these SNPs was observed, but our data are in agreement with results in a U.S.[20] and an Australian cohort.[21] Nonreplication of genetic association could be attributed to population diversity, rare alleles, effect size of the allele, or a host of other factors.[28,29] However, these associations tend to be more meaningful when they are supported with functional data relating to the disease.

Recent evidences by Montes et al.[33] have suggested a functional basis of protection for the rs641153 (R32Q) SNP in AMD. The association of AMD with *CFB* abnormalities strongly suggests that the problem is unregulated activation of the alternative pathway, in which *CFB* is a critical factor. The authors have convincingly demonstrated the underlying mechanistic details of the rs641153 SNP in regulating the activity of the alternate complement pathway that may lead to a reduced risk of AMD.[33] But, since the association of the *CFB* (rs641153) is directly linked to *C2* (rs547154), it would also be interesting to know the combined functional effect of both these SNPs involving the classic pathway.

In summary, we have provided an independent replication of the association of *C2* and *CFB* in an Indian AMD cohort. These results mimic our previous associations with respect to *CFH* and *LOC387715* variants, wherein, our data resembled a similar genetic profile as observed in Caucasian populations.[25,26] Haplotype analysis further refined the region of association harboring the rs547154 and rs641153 SNPs in *C2* and *CFB*. Although our sample sizes were relatively smaller than those from Caucasian populations, these associations underscore the role of *C2* and *CFB* SNPs in AMD pathogenesis and could be used for risk assessment in the Indian cohort.

Acknowledgments

The authors thank all the patients and volunteers for their participation in the study; Nazimul Hussain, Anjli Hussain, Taraprasad Das, Subhadra Jalali, and Avinash Pathangay for providing us some of the initial patient samples; and Kollu N. Rao and Avid Hussain for technical support.

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