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Variation in factor B (*BF*) and complement component 2 (*C2*) genes is associated with age-related macular degeneration

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Abstract

Age-related macular degeneration (AMD) is the most common form of irreversible blindness in developed countries[1,2]. Variants in the factor H gene (*CFH*, also known as *HF1*), which encodes a major inhibitor of the alternative complement pathway, are associated with the risk for developing AMD[3–8]. Here we test the hypothesis that variation in genes encoding other regulatory proteins of the same pathway is associated with AMD. We screened factor B (*BF*) and complement component 2 (*C2*) genes, located in the major histo-compatibility complex class III region, for genetic variation in two independent cohorts comprising ~900 individuals with AMD and ~400 matched controls. Haplotype analyses identify a statistically significant common risk haplotype (H1) and two protective haplotypes. The L9H variant of *BF* and the E318D variant of *C2* (H10), as well as a variant in intron 10 of *C2* and the R32Q variant of *BF* (H7), confer a significantly reduced risk of AMD (odds ratio = 0.45 and 0.36, respectively). Combined analysis of the *C2* and *BF* haplotypes and *CFH* variants shows that variation in the two loci can predict the clinical outcome in 74% of the affected individuals and 56% of the controls. These data expand and refine our understanding of the genetic risk for AMD.

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Note: Supplementary information is available on the Nature Genetics website.

AUTHOR CONTRIBUTION STATEMENT

The AMD Genetics Clinical Study Group includes Stanley Chang, Lawrence A. Yannuzzi, John C. Merriam and Irene Barbazetto (Department of Ophthalmology, Columbia University, New York); Leonid E. Lerner (F.M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania School of Medicine, Philadelphia) and Stephen Russell, Jamal Hoballah, Jill Hageman and Heather Stockman (Department of Ophthalmology and Visual Sciences, Center for Macular Degeneration, University of Iowa, Iowa City, Iowa, USA).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

Inflammation has a central role in the pathobiology of AMD[9–14]. Dysfunction of the complement pathway is proposed to induce significant damage to macular cells, leading to atrophy, degeneration and the elaboration of choroidal neovascular membranes[3,15–17]. Activation of the alternative pathway is initiated by cleavage of C3b-bound factor B (BF), resulting in the formation of the C3Bb complex (C3 convertase). This complex is stabilized by properdin, whereas its dissociation is accelerated by regulatory proteins, including complement factor H (CFH), the major inhibitor of the alternative complement pathway. As *CFH* haplotypes are associated with AMD[3], we hypothesized that the same may be true for activators of the same pathway, such as complement factor B (BF). *BF* and complement component 2 (*C2*), an activator of the classical complement pathway, are paralogous genes located only 500 bp apart on human chromosome 6p21. These two genes, along with genes encoding complement components 4A (*C4A*) and 4B (*C4B*), reside in the major histocompatibility complex (MHC) class III region.

We scanned all 18 *BF* exons in 180 affected individuals and controls from a cohort evaluated at Columbia University. We identified 17 sequence variants; the L9H and R32Q alleles were more frequent in controls than in affected individuals (Table 1). We identified haplotype-tagging SNPs (htSNPs) within *BF* and *C2* (Fig. 1) and genotyped them in 548 affected individuals and 275 controls. Four variants were significantly associated with AMD; the L9H variant in *BF*, which is in nearly complete linkage disequilibrium (LD) with the E318D variant in *C2*, is highly protective for AMD ($P = 0.00020$, odds ratio (OR) = 0.37 (95% confidence interval (c.i.) = 0.18–0.60)). The R32Q allele in *BF* is in nearly complete LD with the rs547154 SNP in intron 10 of *C2* and is also highly protective ($P = 6.43 \times 10^{-9}$, OR = 0.32 (95% c.i. = 0.21–0.48)).

Genotyping of an independent cohort of 350 cases and 114 controls from the University of Iowa confirmed these findings. For example, the *C2* E318D/*BF* L9H SNP pair was significantly associated with AMD ($P = 0.0012$). Haplotypes across the *C2* and *BF* loci were analyzed on the basis of data derived from the combined cohorts (Table 2). The common haplotype (H1, Fig. 1) conferred a significant risk for AMD ($P = 0.0013$). The haplotype tagged by the *BF* R32Q SNP (H7) was highly protective for AMD ($P = 2.1 \times 10^{-7}$) and the haplotype containing the *C2* E318D/*BF* L9H pair (H10) was also significantly protective ($P = 3.4 \times 10^{-6}$). We obtained even more significant results when using the H1 haplotype as a reference OR = 0.42 (95% c.i. = 0.32–0.58) for H7, and OR = 0.33 (95% c.i. = 0.21–0.52) for H10. Individuals with two protective haplotypes (either homozygous for H7 or H10 or 7/10 compound heterozygotes) were found in 3.4% of the controls but in only 0.77% of the affected individuals (OR = 0.22; 95% c.i. = 0.087–0.56). The odds ratio of individuals with two protective alleles was approximately half that of the individuals with one protective allele, consistent with a codominant model.

These associations were statistically significant (Table 2) when the entire AMD cohort was compared with controls, or when major subtypes of AMD, including early AMD and choroidal neovascularization, were analyzed separately. The geographic atrophy (GA) group (133 individuals in two cohorts) deviated from the general trend, similar to our observations with *CFH*[3]. Specifically, haplotype H7 demonstrated the strongest protection against the disease (OR = 0.22) when we compared the GA group with controls, in contrast to OR = 0.45 for the remainder of the AMD subjects. Although this deviation may be important in terms of varying etiology of the disease, it did not reach statistical significance, probably owing to the small number of GA cases.

We initially performed combined analyses with *CFH* by stratifying the subjects according to status at the *CFH* Y402H allele. Protection conferred by *C2* and/or *BF* was strongest in *CFH* 402H homozygotes (OR = 0.27), intermediate in 402H/Y heterozygotes (OR = 0.36) and

weakest in 402Y homozygotes (OR = 0.44). However, the confidence intervals of all these estimates overlapped. The effect is due to a trend in which the frequency of *C2* and/or *BF* protective alleles is greatest in 402H homozygotes; 40% of these individuals in the control cohort carried at least one protective allele. In contrast, controls that were 402H/Y or 402Y had lower frequencies of *C2* and/or *BF* protection (32% and 26%, respectively). That is, individuals at high risk owing to their *CFH* genotype who have not developed AMD have a high frequency of protective allele(s) at the *C2/BF* locus.

To identify the possible combinations of *CFH* and *C2*/*BF* SNPs that are protective for AMD, we analyzed the data with an empirical model and then with a machine-learned model using Exemplar software (Fig. 2). The first model was a hypothesized (hand-built) model, such as one would create by an empirical inspection of the data (Fig. 2a). The model yields four possible combinations of genotypes that protect from AMD (that is, combinations that result in the model being 'true'). We applied this model to the samples (Fig. 2b) and subjected the resulting distributions to significance testing (Fisher's exact test; $P = 0.00237$, $P = 4.28 \times 10^{-8}$ and $P = 7.90 \times 10^{-10}$ for the Iowa, Columbia and combined cohorts, respectively). Subsequently, Exemplar software generated a protective model that provided a 'best fit' to the data using a machine-learning method called Genetic Algorithms to test the hypothesis that the machine-learning software can outperform the hand-built model. Models were learned on the Columbia cohort, and the resulting fittest models were applied to the Iowa cohort (for out-of-sample verification) and to the combined sample set. The best-performing model (Fig. 2c,d) describes four possible genotype combinations that protect from AMD ($P = 7.49 \times 10^{-5}$, $P = 2.97 \times 10^{-22}$ and $P = 1.69 \times 10^{-23}$, for the Iowa, Columbia and combined cohorts, respectively). We further validated this method by randomizing the case and control designations and performing 3,000 permutations of the dataset. In summary, combined analysis of these haplotypes with the variation in *CFH* showed that 56% of unaffected controls harbor at least one protective *CFH* or *C2*/*BF* haplotype, whereas 74% of AMD patients lack any protective haplotype. Approximately 60% of the risk in affected individuals and 65% of the protection of controls are due to the *CFH* locus, and the remainder (40% and 35%, respectively) to the *C2*/*BF* locus. The machine-learned model outperformed the hand-built model, providing more accurate predictions of clinical outcome in cases and controls. An analysis with a classification and regression tree (C&RT) method was used to independently confirm the relative contribution of the *CFH* and *C2*/*BF* loci in AMD. The C&RT models estimate that *C2* and *BF* alleles account for 27–37% of the cases, which is consistent with the 35–40% estimated contribution of the *C2*/*BF* locus from the Genetic Algorithm analysis (see Supplementary Methods online for details).

BF and *C2* are expressed in the neural retina, RPE and choroid. We generated RT-PCR products for the *BF* and *C2* gene from RPE, RPE/choroid complex and neural retina derived from eyes from donors with AMD (ages 67 and 94 years) and without AMD (ages 69 and 82 years) (data not shown). BF protein was present in ocular drusen and Bruch's membrane and less prominently in the choroidal stroma (Fig. 3a). Ba (a BF-derived peptide) immunoreactivity was less pronounced but distinctly present in patches associated with RPE cells and throughout Bruch's membrane (Fig. 3b). The distribution of BF was similar to that of C3 (Fig. 3c), both of which are essentially identical to that of CFH and C5b-9 (ref. [3]).

In summary, these data show that variants in the complement pathway–associated genes *C2* and *BF* are significantly associated with AMD. Protective haplotypes in the *C2*/*BF* locus contain nonsynonymous SNPs in the *BF* gene, an important activator of the alternative complement pathway. Available data confirms the hypothesis that the AMD phenotype may be modulated by abnormal BF activity. Indeed, the BF protein containing glutamine at position 32 (resulting from one of the two *BF* SNPs tagging a protective haplotype) has been

shown to have reduced hemolytic activity as compared with the more frequent Arg32 form[18]. Notably, the same study did not document a functional effect for the R32W variant, which was not associated with AMD in the current study. On the basis of these data, we suggest that an activator with reduced enzymatic activity may provide a lower risk for chronic complement response that can lead to drusen formation and AMD. This hypothesis is compatible with our previous proposal that insufficient inhibition of the alternative complement cascade owing to variation in *CFH* results in chronic damage at the retinal pigment epithelium/Bruch's membrane interface[3,9,10]. Another *BF* htSNP, L9H, resides in the signal peptide. Although the functional consequence of this variant has not been demonstrated directly, this variant could conceivably modulate BF secretion.

The genetic and functional data suggest that variation in *BF* is probably causal for the observed association with AMD. The two haplotype-tagging variants in *BF* are nonconservative, and one of the two is documented to have a direct functional relevance, whereas the variants in C2 are a conservative change and an intronic SNP. In addition, BF participates directly in the alternative pathway, a pathway that also involves CFH. We cannot rule out a direct role for *C2*, however, particularly because both C2 and BF regulate the production of C3. C2 and BF have nearly identical modular structures, including serine protease domains and three complement control protein (CCP) modules. Additional support for the involvement of *BF* in AMD pathogenesis comes from studies of drusen composition. Although the majority of proteins involved in the alternative pathway (CFH, BF, *etc*.) are found in drusen, their analogs from the classical pathway, such as C2 and C4, are not[11,13] (G.S.H., unpublished data). These data further suggest that the C2 SNPs are associated with AMD owing to extensive LD with BF.

Several common functional variants in both *C2* and *BF* have been described[19–21], but most of these are rare. We analyzed all missense alleles with frequencies >2% in European populations as judged from resequencing data. Moreover, no additional nonsynonymous variants in either gene have been found after complete sequencing of several MHC haplotypes, including examples of our haplotypes H2, H5, and H7 (ref. [22]).

Because *C2* and *BF* reside in the MHC locus with many other genes involved in inflammation, it is possible that the associations observed in this study are due to LD with adjacent loci[23]. Five lines of evidence, however, suggest that the *C2*/*BF* locus is the main contributor to the observed association. First, there is only modest LD between *C2*/*BF* and adjacent class III loci (Supplementary Fig. 1 online). Second, MHC class II loci and *BF* haplotypes H7 and H10 do not show strong LD (G.S.H., unpublished data). Third, in a whole-genome scan[6], the MHC locus did not demonstrate a statistically significant association with AMD. That analysis included 80 SNPs across the MHC locus but did not include any of the eight SNPs typed in this study. Fourth, estimated recombination rates from HapMap data indicate regions of high recombination on both sides of the C2/BF locus[24] (M.D. and B.G., unpublished data). Finally, the single published study on MHC in AMD demonstrates modest protection for the class I locus B*4001 ($P = 0.027$) and the class II locus DRB1*1301 ($P = 0.009$)[25]. Because the protective alleles identified in this study are associated with AMD at a substantially higher statistical significance, it is very unlikely that the *C2*/*BF* association is due to LD with these and/or other loci in the MHC.

In conclusion, this study extends and refines the role of the alternative complement pathway in the pathobiology of AMD and further strengthens the proposed model that inflammation, infection or both have a major role in this common ocular disease.

METHODS

Patients

Two independent groups of individuals affected with AMD and age-matched controls of European-American descent over the age of 60 were used in this study. These groups consisted of 350 unrelated individuals with clinically documented AMD (mean age 79.5 ± 7.8 years) and 114 unrelated control individuals (mean age 78.4 ± 7.4 years; matched by age and ethnicity) from the University of Iowa and 548 unrelated individuals with clinically documented AMD (mean age 71.32 ± 8.9 years) and 275 unrelated age- and ethnicity-matched controls (mean age 68.84 ± 8.6 years) from Columbia University. Patients were examined by trained ophthalmologists.

Stereo fundus photographs were graded according to standardized classification systems as described previously[3,26,27]. Controls did not show any distinguishing signs of macular disease nor did they have a known family history of AMD (stages 0 and 1a). AMD patients were subdivided into phenotypic categories on the basis of the classification of their most severe eye at the time of their recruitment. Genomic DNA was generated from peripheral blood leukocytes using QIAamp DNA Blood Maxi kits (Qiagen).

Studies were conducted under protocols approved by the Institutional Review Boards of Columbia University and the University of Iowa. Informed consent was obtained from all study subjects before participation.

Immunohistochemistry

Posterior poles were processed, sectioned and labeled with antibodies directed against factor B (Quidel), as described previously[9]. Adjacent sections were incubated with secondary antibody alone to serve as controls. Some immunolabeled specimens were prepared and viewed by confocal laser scanning microscopy, as described[9].

Mutation screening and analysis

Coding and adjacent intronic regions of *BF* and *C2* were examined for variants using SSCP analyses, denaturing high performance liquid chromatography (DHPLC) and direct sequencing. Primers for SSCP, DHPLC and DNA sequencing analyses were designed to amplify each exon and its adjacent intronic regions using MacVector. Primer sequences are available upon request. PCR-derived amplicons were screened for sequence variation, as described earlier[28,29]. All changes detected by SSCP and DHPLC were confirmed by bidirectional sequencing according to standard protocols.

Genotyping

SNPs were discovered through data mining (Ensembl database, dbSNP, Celera Discovery System) and through sequencing. Assays for variants with >10% frequency in test populations were purchased from Applied Biosystems as Validated, Inventoried SNP Assays-On-Demand or were submitted to an Applied Biosystems Assays-By-Design pipeline. The technique used was identical to that described previously[3]. Briefly, 5 ng of DNA were subjected to 50 cycles on an ABI 9700 384-well thermocycler, and plates were read in an Applied Biosystems 7900 HT Sequence Detection System.

Statistical analysis

Genotypes were tabulated in Microsoft Excel and presented to SPSS for contingency table analysis as described previously[3,27]. Compliance to Hardy-Weinberg equilibrium was checked using SAS/Genetics (SAS Institute), and all SNPs in both cases and controls

survived a cutoff of $P < 0.05$. For haplotype estimation we used snphap (written by David Clayton, Cambridge Institute for Medical Research), SNPEM (written by Nicholas Schork (University of California, San Diego) and M. Daniele Fallin (Johns Hopkins University)) and PHASE version 2.11 (Matthew Stephens, University of Washington, Seattle). The haplotype analysis strategy was, first, to obtain haplotype estimates using the Expectation Maximization (EM) or Gibbs sampling algorithm; second, to identify htSNPs representing a minimal informative set within a region of linkage disequilibrium and third, to assess these for significant association with AMD. All *P*-values are two-tailed, and X^2 values are presented as asymptotic significance. Overall type I error rates (α) were retrospectively calculated using a previously described method[30] and are below 2×10^{-3}. Significant haplotypes were subjected to permutation testing in both SNPEM and PHASE. The protective SNP model in Figure 2a was presented to Exemplar 2.2 and statistically evaluated by that software for fitness against the three datasets (Iowa, Columbia and combined) in Figure 2b. Generation of the genetic algorithm (GA)-derived model (Fig. 2c) involved Exemplar software. The GA options were set to 1,500 AND/OR models of 15 iterations each, with a model size no larger than 5 (which permits 16 possible genotypes). Further details of the genetic algorithm implementation and significance testing are available in the Supplementary Methods.

A Classification & Regression Tree Analysis was performed with SPSS (v 14.0) with the appropriate module on the Columbia, Iowa and combined data recoded as with (+) or without (−) minor alleles. Models were automatically generated using each of the three data sets that incorporated both *CFH* and *C2*/*BF* loci as contributors to the dependent outcome.

Accession codes

GenBank: complement factor B (properdin, BF): mRNA, AF349679; protein, AAK30167. Complement component 2 (C2): mRNA, AK222537; protein, BAD96257. dbSNP identification numbers for genotyped SNPs are provided in Table 2.

URLs

Resequencing data used for analysis of all missense alleles with frequencies >2% in European populations is available at http://pga.mbt.washington.edu/. The list of SNPs included on the Affymetrix Mapping 100K Array is available at https://www.affymetrix.com/analysis/netaffx/index.affx. snphap is available from http://www-gene.cimr.cam.ac.uk/clayton/software/. PHASE version 2.11 is available at http://www.stat.washington.edu/stephens/software.html. Linkage disequilibrium was assessed using the graphical tools available at http://www.innateimmunity.net. Overall type I error rates (α) were calculated using a previously described method[30] implemented at https://innateimmunity.net/IIPGA2/Bioinformatics/multipletestfdrform. Exemplar 2.2 is available at http://www.sapiosciences.com. Further details of the genetic algorithm implementation and significance testing are available at http://www.sapiosciences.com/papers/AMDSupplement.pdf.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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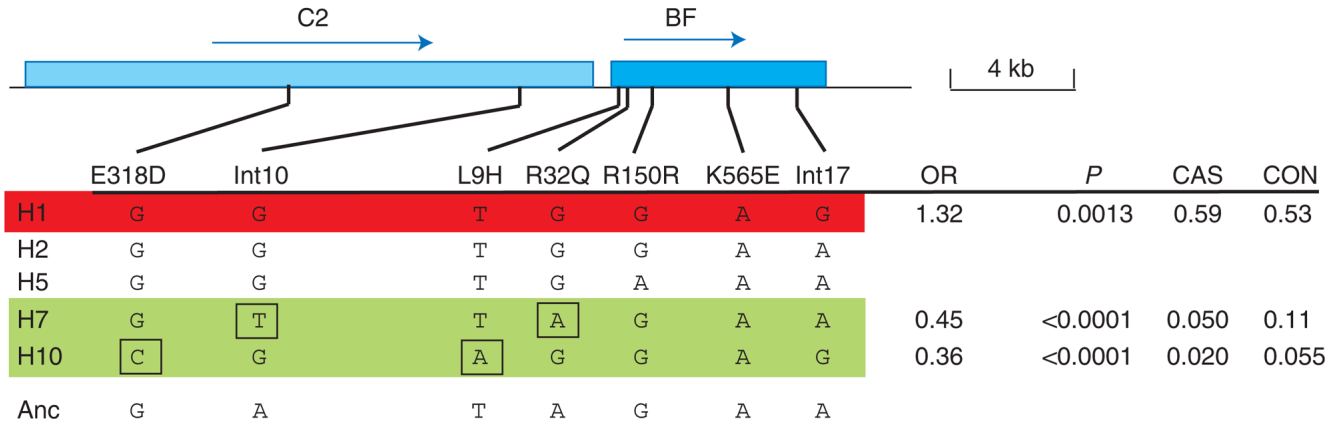

Figure 1.

Diagram and haplotype analysis of SNPs in *BF* and *C2*. The SNPs used in the study are shown along with the predicted haplotypes, odds ratios (OR), *P* values and frequencies in the combined analysis of affected individuals (CAS) and controls (CON). The 95% c.i. for H7 is 0.33–0.61 and for H10 is 0.23–0.56. The ancestral (chimpanzee) haplotype is designated as Anc. Examples of haplotype H2 (AL662849), H5 (AL645922, NG_004658) and H7 (NG_000013) have been sequenced, and no additional nonsynonymous variants in either the *C2* or *BF* genes are present[22].

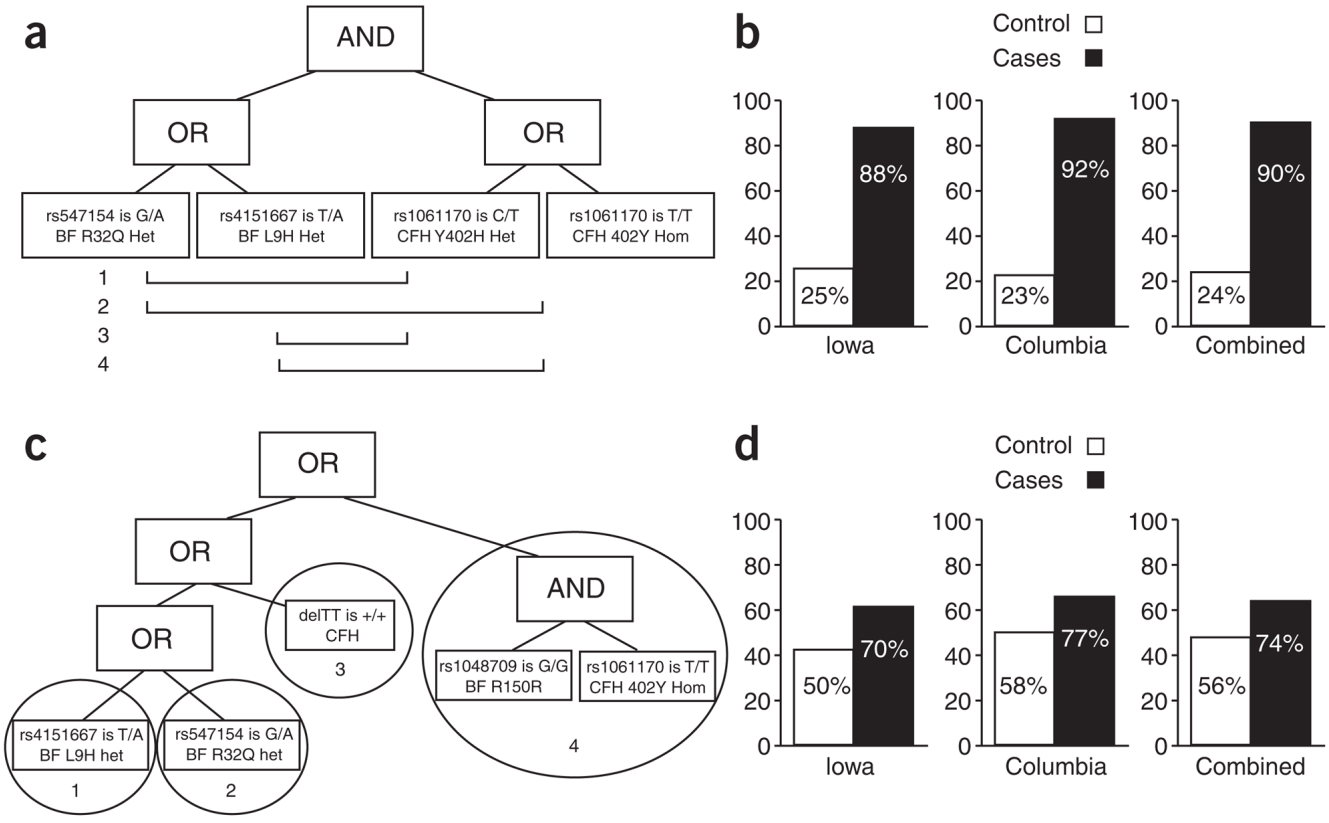

Figure 2.

Combined complement gene analyses. Individual SNP analyses suggested several possible combinations of SNPs that protect an individual from developing AMD. To test these, an empirical model was applied first. (**a**,**b**) Fraction accounted for by hypothesized model. The model gives four possible combinations of genotypes (**a**) that would protect from AMD: (i) rs547154 (R32Q) is G/A AND rs1061170 (Y402H) is C/T, (ii) rs547154 is G/A AND rs1061170 is C/C, (iii) rs4151667 (L9H) is T/A AND rs1061170 is C/T, and (iv) rs4151667 is T/A AND rs1061170 is C/C. Application of this model resulted in the distributions shown in **b** for the Iowa, Columbia and combined cohorts. The case percentage is the percentage of cases for which the model was false; that is, they did not have protection as described by the model. The control percentage is the percentage of controls that did have the protective factors. (**c**,**d**) Fraction accounted for by the Exemplar machine-built model. **c** shows the best-performing model, which describes four possible individual or combinations of genotypes that protect from AMD (that is, combinations resulting in the model being ‘true’): (i) rs1048709 (R150R) is G/G AND rs1061170 is C/C, OR (ii) rs547154 is G/A, OR (iii) rs4151667 is T/A OR (iv) CFH intron 1 variant is delTT. The model performance is shown in **d** for the Iowa, Columbia, and combined cohorts.

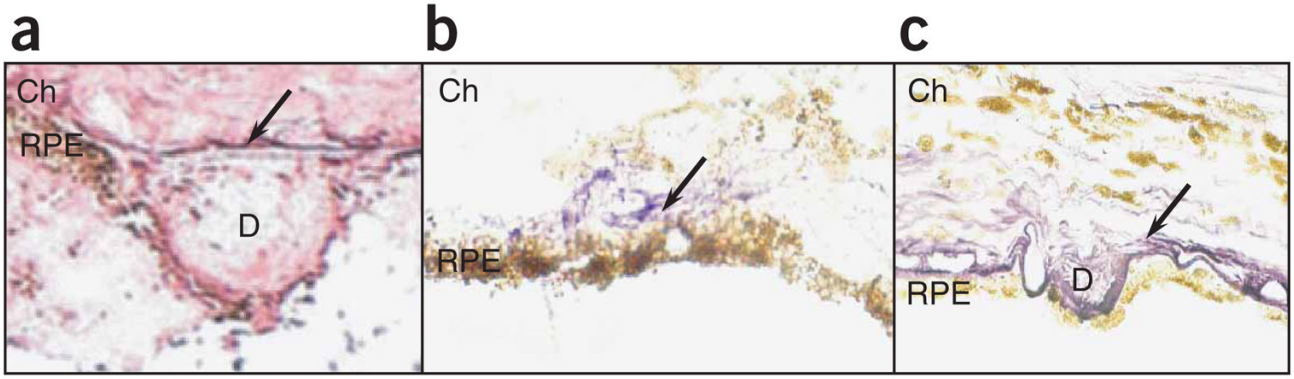

Figure 3.

Immunolocalization of proteins along the retinal pigment epithelium (RPE)-choroid (Ch) complex in sections from an unfixed eye of a 72-year-old donor with early-stage AMD. (**a**) BF. (**b**) Ba (a fragment of the full-length factor B). (**c**) C3. Anti-BF (red) labels drusen (D), particularly along their rims, Bruch's membrane and the choroidal stroma. Anti-Ba (purple) labels Bruch's membrane and RPE-associated patches. The distribution of BF is similar to that of C3. Brown coloration in the RPE cytoplasm and choroid is due to melanin.

Table 1

Sequence variants in the *BF* gene detected by DHPLC screening

			Allele frequency in affected individuals				
Exon	**Nucleotide change**	**Amino acid change**	**AMD total**	**N**	**GA**	**E**	**Allele frequency in controls**
1	26 T→A	L9H	18/1092	10/546	2/178	6/368	23/546
2	94 C→T	R32W	109/1096	52/546	20/182	37/368	55/550
2	95 G→A	R32Q	44/1096	21/546	4/182	19/368	61/550
3	405 C→T	Y135Y	1/184	1/184			0/184
4	504 G→A	P168P	4/184	4/184			6/184
4	600 C→T	S200S	0/184	0/184			2/184
5	673 C→T	Y252Y	2/184	2/184			5/184
5	754 G→A	G252S	7/184	7/184			6/184
6	897+17C→A		2/184	2/184			1/184
8	1137 C→T	R379R	1/184	1/184			0/184
9	1169-35T→A		1/184	1/184			0/184
12	1598 A→G	K533R	3/184	3/184			9/184
14	1693 A→G	K565E	9/182	9/182			4/184
14	1697 A→C	E566A	9/182	9/182			4/184
15	1856-14C→T		13/182	13/182			21/184
15	1933 G→A	V645I	1/182	1/182			0/184
18	*23 C→T		4/182	4/182			7/182

Subclasses of AMD: N, neovascular; GA, geographic atrophy; E, early.

Table 2

Association analysis of *C2*/*BF* variants in combined Columbia and Iowa cohorts

Gene	refSNP ID	Location	No. of affected individuals	No. of controls	X^2	*P*	OR	95% c.i.
C2	rs9332739	E318D	897	381	21.2	4.14×10^{-6}	0.36	0.23–0.56
C2	rs547154	IVS 10	894	382	28.7	8.45×10^{-8}	0.44	0.33–0.60
BF	rs4151667	L9H	903	383	21.3	3.93×10^{-6}	0.36	0.23–0.56
BF	rs641153	R32Q	551	269	33.7	6.43×10^{-9}	0.32	0.21–0.48
BF	rs1048709	R150R	892	381	0.12	NS		
BF	rs4151659	K565E	902	384	1.1	NS		
BF	rs2072633	IVS 17	893	379	4.05	0.044	0.84	0.70–0.99

NS, not significant.

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