Extended Haplotypes in the Complement Factor H *(CFH)* and CFH-related *(CFHR)* Family of Genes that Protect against Age-related Macular Degeneration: Identification, Ethnic Distribution and Evolutionary Implications

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Abstract

Background—Variants in the complement factor H gene *(CFH)* are associated with age-related macular degeneration (AMD). *CFH* and five *CFH*-related genes *(CFHR1-5)* lie within the regulators of complement activation (RCA) locus on chromosome 1q32.

Aims and Methods—In this study, we refined the structural and evolutionary relationships between these genes and AMD using a combined molecular and immunohistochemical approach.

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Key Messages:

A large, common deletion that encompasses both the complement Factor H-related 1 *(CFHR1)* and complement Factor H-related 1 *(CFHR3)* genes is observed in 1.1% of AMD cases and 5.7% of matched controls (χ^2=32.8; P=1.6 E-09), indicating that the absence of one or both of these two genes may account for the protective effects conferred by some *CFH* haplotypes.

The *CFHR1/CFHR3* deletion is commonly found in many different populations in the world -- an indication of its ancient origin.

The extremely low, practically nonexistent, levels of *CFHR1* and *CFHR3* transcription by the ocular RPE/choroid complex and neural retina suggest that functions ascribed to the respective proteins are mediated through hepatic synthesis and subsequent release into the circulation.

Results—We identify and characterize a large, common deletion that encompasses both the *CFHR1* and *CFHR3* genes. CFHR1, an abundant serum protein, is absent in subjects homozygous for the deletion. Analysis of AMD cases and controls from two cohorts demonstrates that deletion homozygotes comprise 1.1% of cases and 5.7% of the controls (2=32.8; P=1.6 E-09). *CFHR1* and *CFHR3* transcripts are abundant in liver, but undetectable in the ocular RPE/choroid complex. AMD-associated *CFH/CFHR1/CFHR3* haplotypes are widespread in human populations.

Conclusion—The absence of *CFHR1* and/or *CFHR3* may account for the protective effects conferred by some *CFH* haplotypes. Moreover, the high frequencies of the 402H allele and the *delCFHR1/CFHR3* alleles in African populations suggest an ancient origin for these alleles. The considerable diversity accumulated at this locus may be due to selection, which is consistent with an important role for the CFHR genes in innate immunity.

Introduction

As the leading cause of irreversible vision loss in the developed world, age-related macular degeneration (AMD) affects an estimated 25% of individuals over the age of 75 (1,2). A host of studies conducted over the past decade has provided compelling biological evidence that immune-mediated processes and aberrant regulation of the complement cascade's alternative pathway contribute to the etiology of AMD (3–8). Definitive support for this new AMD paradigm has emerged from recent genetic studies that have shown a highly significant association between AMD and specific haplotypes of the complement factor H gene (*CFH*), which lies within the "regulators of complement activation" (RCA) gene cluster on chromosome 1q25-q31 (9–13). Further support for the involvement of the complement cascade is provided by a related discovery that haplotypes of two additional complement components, factor B *(BF)* and complement component 2 *(C2)* – which lie within the major histocompatibility (MHC) class III region on 6p21 -- confer protection or increase the risk for AMD. Combined analyses of the *CFH* and *BF/C2* variants predicts clinical outcome in approximately 74% of AMD cases (14).

Data related to the association of *CFH* with AMD in the Caucasian and Indian populations is consistent (15–28). Several recent reports, however, suggest that risk variants of the *CFH* gene in the Japanese and Chinese populations are either not associated with AMD, differ substantially from the Caucasian population and/or that the risk haplotype occurs at a substantially lower frequency (29–31). Although they are based upon relatively small cohorts, these reports indicate that additional genetic analyses are warranted across a wide variety of ethnic groups.

The *CFH* gene consists of 20 short consensus repeats (SCR), each of which encodes a functional domain of approximately 60 amino acids (32,33). In addition to the full-length transcript, there is an alternatively-spliced, truncated gene product of *CFH (CFHT)* containing SCRs 1–7. Five additional members of the *CFH* gene family, represented by five separate genes, are designated as *CFH*-related *(CFHR)* 1–5. Each of the five CFH-related proteins contains a set of SCR domains, many of which are homologous to those in CFH (Figure 1) (34). Most notably, the two most C-terminal SCR domains are highly conserved within the CFH-protein family. These domains contain a C3d/C3b binding site and the hot-spot for mutations in the atypical hemolytic uremic syndrome (35). All members of the Factor H gene

family appear to be synthesized primarily by cells in the liver, secreted, and then released into the circulation (33,36).

Although our understanding of nucleotide polymorphisms in loci associated with AMD has increased substantially, especially in the case of CFH, characterization of the disease-associated *CFH* risk and protective haplotypes has not yet been assessed fully. In this study, we identify AMD-associated haplotypes spanning *CFH* and *CFHR1-5*. We define a large deletion encompassing two *CFH*-related genes -- *CFHR1* and *CFHR3* – that further refines our understanding of the protection conferred by the *CFH* haplotypes identified previously (9). We also determine the frequency of a major *CFH* risk allele (Y402H) within numerous human populations. Based on these analyses, we propose a hypothesis to explain the evolution of genetic variation in the *CFH* locus, with a focus on the development of AMD.

Materials and Methods

Patients and Donors

Three independent cohorts of AMD cases and AMD-unaffected, age-matched controls were used for this study. Individuals in two cohorts were of European-American descent and those of the third cohort of Finnish decent; all individuals were over the age of 60, unrelated and matched for age and ethnicity. All patients were enrolled under Institutional Review Board-approved protocols. These groups consisted of 583 patients/donors with clinically documented AMD and 370 control individuals/donors from the University of Iowa; 635 patients with clinically documented AMD and 357 controls from Columbia University; and 46 patients with clinically documented AMD and 33 unrelated controls from Helsinki University. Patients were examined and photographed by trained ophthalmologists. Stereo fundus photographs were graded according to standardized classification systems (37,38) as described previously (9, 28). Fundi and/or posterior poles of human donor eyes were graded using the same criteria.

Population frequencies of the CFH Y402H variant and the CFHR1/CFHR3 deletion were assessed using samples from the Human Genome Diversity Panel, which is composed of 1064 individuals from 47 defined ethnic groups (39).

Polymerase Chain Reaction (PCR)

Total genomic DNA was prepared from blood using a QIAamp DNA Blood Kit (Qiagen, Valencia, CA). Identification of *CFHR1-5* DNAs were made using polymerase chain reaction (PCR). PCR reactions utilized 10 ng human genomic DNA in the manufacturer's 1X NH4 buffer, 2.5 mM MgCl2, 1 mM deoxynucleotide triphosphate (dNTP) mix and 10–25 uM of the respective primer pairs. All PCR reactions were performed in a Hybaid thermocycler. Reaction parameters were one cycle at 94°C for 3 minutes, 40 cycles at 94°C for 45 sec, 55.3°C *(CFH)*/55.3°C *(CFHR1)*/49.4°C *(CFHR2)*/50°C *(CFHR3)*/49.4°C *(CFHR4)*/49.4°C *(CFHR5)* for 1 min, 72°C for 1 min and one cycle at 72°C for 3 min. The PCR products were run on 2% agarose gels and imaged using a Gel Doc 2000™ Documentation System.

Mutation Screening and Analysis

CFH, *CFHR1* and *CFHR3* were assessed using single strand confirmation polymorphism (SSCP) analyses, denaturing high performance liquid chromatography (DHPLC) and direct sequencing as described previously (9). Primers for SSCP, DNA PCR amplification and DNA sequencing analyses (Supplemental Table 1) were designed to amplify each exon and its adjacent intronic regions using MacVector software (San Diego, CA). Statistical analyses, including chi-square and Fisher's exact tests, were performed as described previously (14).

Haplotype Network Analysis

Haplotypes were inferred using PHASE 2.1 (40,41) choosing the best fit to the European American data as source input for the network. Rare haplotypes (representing less than 0.5% of those inferred) were discarded before input into Network 4.2 (Flexus Engineering, see: http://www.fluxus-engineering.com/sharenet.htm). Ancestral sequences were chosen by comparison of the Human May 2004 assembly (hg 17, Build 35) at University of California Santa Cruz (http://genome.ucsc.edu) with November 2003 Chimpanzee sequence. A Reduced Median network (42) was visualized first, followed by a Median Joining network (43) and a median joining network with star contraction (44). Each network produced similar findings for the ancestral haplotype and distance from protective and susceptible haplotypes.

Western Blot Analyses

Human serum samples were collected in red-topped tubes, centrifuged, processed within one hour of collection and stored at −80°C. Serum samples from 65 Iowan and 79 Finnish patients were diluted to a final dilution of 1:200. 4 ul of diluted serum was loaded on 4–20% gradient gels and the proteins separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA). The membranes were blocked in PBS buffer containing 5% non-fat milk for 60 min at room temperature. Polyclonal goat anti-factor H antiserum (Calbiochem-Novabiochem Corp. San Diego, California, USA) was diluted in blocking buffer at a concentration of 1:2000. After incubation at 4ºC for 16 hrs, the membranes were washed four times in PBS followed by incubation with a rabbit polyclonal anti-goat immunoglobulin G conjugated to horse peroxidase (IgG HRP; Abcam, Cambridge, MA) in blocking buffer at a dilution of 1:50000 for 40 min at room temperature. The membrane was washed four times with Tris-buffered saline containing 0.05% Tween 20 (TBST) prior to developing with Supersignal Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Human Donor Eyes

Human donor eyes and liver were obtained from the Iowa Lions Eye Bank within five hours of death. Total RNA for RT-PCR and QPCR analyses was isolated from the RPE/choroid and retinal punches of eight donors using Qiagen RNeasy mini-preps (Qiagen, Valencia, CA) according to the manufacturer's suggested protocol. Contaminating DNA was digested with RNAse-free DNAse and the RNA was re-purified using a second RNeasy mini-column. RNA integrity was assessed by microchannel electrophoresis on an Agilent Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

Quantitative RT-PCR

Real-time quantitative polymerase chain reaction (QPCR) was carried out using the SYBR Green method (45). Gene-specific primers were designed using Beacon Design 4.0 (Premier Biosoft International, Palo Alto, CA) and the sequences of the PCR primers used are listed in Supplemental Table 1.

cDNA was synthesized from 150 to 640 ng of DNAse-treated total RNA using the the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Aliquots representing 1/25th of the resulting cDNA were employed in triplicate 20 μl PCR reactions using the following reaction conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTP's, 0.20 units Platinum Taq DNA polymerase, 1X SYBR Green (Invitrogen Corp., Carlsbad, CA), and 500 nM sense and anti-sense gene-specific primers. Amplification was carried out using the following temperature profile: 3 min at 94°C followed by 45 cycles of 15 sec at 93°C, 15 sec at 56°C, 90 sec at 72°C, 20 sec at 78°C, and 20 sec at 82°C. Real-time fluorescence readings were made at 72°C, 78°C, and 82°C. The specificity of amplification

was ascertained by melting profile analysis and gel electrophoresis. For each of the CFH gene family members the analysis was carried out using the measurements made at 78°C. The housekeeping genes (GAPD, GPI, HMBS, and HPRT1) were quantified using the 82°C measurements. The efficiency of the PCR reaction was determined by standard curve analysis using serial dilutions of cDNA prepared from total human liver RNA (Origene Technologies, Inc., Rockville, MD), and the level of expression was described by the equation: amount = $1/(1+E)^{Ct}$, where E is the efficiency and Ct is the PCR cycle number at which the fluorescent signal crosses a predetermined threshold while still undergoing exponential amplification. For each primer set the threshold was set proportional to the size of the amplicon to adjust for differences in signal intensities due to product length. The *CFH* gene family data was normalized to the geometric mean of two "housekeeping" genes [glyceraldehyde phosphate dehydrogenase (*GAPD*) and glucose phosphate isomerase (*GPI*)] whose expression levels were empirically determined to remain constant amongst all tissue types and donors used in this study (46).

Results

Absence of CFHR1 Protein in Sera

CFHR1 is an abundant plasma protein (34,47) that circulates at relatively high concentrations (40 μg/ml). Initial immunoblot analyses of anti-human CFH antibody-reactive protein bands in sera obtained from 25 individuals in the Iowa AMD study cohort revealed that CFHR1 was not detectable in three samples, each of which was derived from control subjects without AMD (two were homozygous for the CFH 1277T allele and the third was heterozygous at this position) (Figure 2). Based on these data, *CFHR1*-specific primers were designed and utilized (Supplemental Table 1) to assess whether the *CFHR1* gene is present in DNA obtained from the same individuals. This analysis indicated that the *CFHR1* gene was deleted in the subjects lacking immunoreactive serum CFHR1 protein.

Identification of A Common Deletion Spanning the CFHR1 and CFHR3 Genes

SSCP analysis and direct DNA sequencing was used to determine the frequency of the homozygous deletion of the *CFHR1* gene in 953 additional individuals in the Iowa cohort (Supplemental Table 2). Homozygous deletions were documented in 2.7% of this cohort. Using a gene-specific PCR assay, the homozygous *CFHR1* deletion was found in 3.0% of study subjects from a cohort ascertained at Columbia University comprised of 992 individuals (Supplemental Table 2).

To determine whether the deletion was associated with AMD, we examined the distribution of the deletion homozygotes in the 576 AMD cases and 352 controls from the Iowa cohort (Table 1). Deletion homozygotes represented 4.9% of the controls and 1.2% of the cases. This analysis was replicated on a cohort of individuals from Columbia University; 6.7% of the controls and 0.94% of the cases were deletion homozygotes in this cohort. The combined data set demonstrated a highly significant protective effect of the *CFHR1* deletion for AMD (2 = 32.8, p = 1.6 X 10–9) with an odds ratio of 0.19 [95%CI 0.099–0.37].

Although individuals heterozygous for the *CFHR1* deletion could not be assessed directly in these analyses, we could reliably score most of the samples through examination of their DNA sequence traces (data not shown). These data showed that *CFHR1* deletion heterozygotes were less common in cases (17%) than in controls (22%), indicating that the deletion in the heterozygous state may also be somewhat protective.

Primers specific for the *CFHR2, CFHR3, CFHR4* and *CFHR5* genes (Supplemental Table 1) were designed to further characterize the extent of the deletion in the RCA locus. The PCR

analyses revealed that the deletion includes the *CFHR3* gene (based on assessment of exon 3), but does not include the *CFHR2, CFHR4* or *CFHR5* genes (Supplemental Figure 1).

Examination of Western blots of serum from an additional 40 Iowa patients revealed that all ten of the *delCFHR1* homozygotes had no detectable CFHR1 protein, whereas the remaining 30 serum samples contained variable amounts of protein (data not shown). These analyses confirmed that *delCFHR1/CFHR3* homozygotes lack the *CFHR1* gene as well as the corresponding protein. Analysis of a Finnish cohort using Western analysis showed that two controls (2.5%; both CFH 1277TT homozygotes) lacked CFHR1-reactive bands (Supplemental Table 3). CFHR1-immunoreactive bands were noted in 83.5% of the samples, whereas 13.8% of the samples (all TT and TC at position 1277) exhibited fainter bands presumably derived from CFHR1 heterozygotes. All of the CC homozygotes at position 1277 exhibited robust immunoreactivity. The frequencies of robust, faint and nonexistent immunoreactive CFHR1 bands closely matched expected Hardy Weinberg equilibrium predictions for this sample set [64 (81%):14 (18%):1 (1.2%)].

Haplotype Analysis of the CFHR1/3 Deletion

Analysis of the combined dataset was performed to determine what *CFH* haplotype(s) contained the *delCFHR1/CFHR3* allele. The deletion was rarely found on the haplotype tagged by the major *CFH* risk allele, 402H (Supplemental Table 4). Approximately 63% of *CFHR1/CFHR3* deletions lie on a previously identified protective *CFH* haplotype (9); however, not every *delCFHR1/CFHR3* chromosome was associated with H4. The protection conferred by *delCFHR1/CFHR3* homozygosity was stronger than that of H4 homozygotes (haplotypes for all of the Iowa *delCFHR1/CFHR3* homozygotes across the *CFH, CFHR1 and CFHR3* genes are depicted in Supplemental Table 5). We conclude from these data that some of the previously described protection associated with the *CFH* H4 haplotype is due to the *delCFHR1/CFHR3*.

Ocular Expression of CFH, CFHR1 and CFHR3

To determine whether *CFHR1* and/or *CFHR3* transcripts are expressed in the RPE/choroid or neural retina, cDNA was synthesized from total RNA obtained from eight donors. Based upon SSCP analysis, two of these were confirmed as *delCFHR1/CFHR3* homozygotes, and the remaining six carried at least one *CFHR1* and one *CFHR3* allele. Triplicate aliquots were subjected to quantitative real-time PCR using primers specific for full length *CFH*, truncated *CFHT*, *CFHR1* and *CFHR3* (see Supplemental Table 1).

No expression of CFHR1 or CFHR3 above background was detected in any of the 8 RPE/choroid samples (Table 3). In four of the donors with at least one *CFHR1* allele, extremely low expression was detected in the retina, but at levels >250,000 fold lower than in liver controls. No expression of CFHR1 in the retina was found in the two *delCFHR1/CFHR3* homozygotes.

In contrast, mean CFH expression by the RPE/choroid in this group was only 10 fold below that of liver. CFH expression in the retina was nearly 300 fold lower than in the RPE/choroid, a level that could be attributable to residual RPE cells that remain adherent to the retina after dissection (Table 3). On average, the ratio of full length to truncated CFH mRNA was similar in both RPE/choroid and liver (1.5 +/− 0.4) for those donors with at least one copy of *CFHR1/CFHR3*. In the two *delCFHR1/CFHR3* homozygotes, the CFHv1/CFHv2 ratios were somewhat higher (2.2 and 3.7).

Haplotype Network Analysis

To analyze the relationship of the *CFH* region haplotypes, including the haplotype(s) associated with the *CFHR1/3* deletion, haplotype network analysis was performed (Figure 3). This method determines the most likely pathway for the evolution of the haplotypes and

displays the predicted haplotype network. A circle that is proportional in size to its abundance depicts the estimated frequency of each haplotype. Haplotype network analysis of the variants in the *CFH* region revealed that the protective H4 and H5 haplotypes are most closely related to the sequence found in the chimpanzee, and therefore closer to the ancestral state. Haplotypes determined to be neutral in regards to their risk for AMD, as well as the haplotype that confers risk for atypical hemolytic uremic syndrome (aHUS) are more divergent from the ancestral haplotype. The haplotype containing a major *CFH* risk allele -- Y402H -- is the most divergent.

Population Frequencies of CFH Y402H and Deletion Alleles

Given the divergence of the *CFH* Y402H-containing haplotype, we determined the distribution of this allele in the Human Genome Diversity Panel (HGDP), a set of 1064 DNA samples from 47 defined populations. The *CFH* 402H allele occurred at a frequency of 35% in both African and European populations, 25–30% in North African and Middle Eastern populations, 10–15% in Asian populations and 0–5% in Native American populations (Table 2). We found extremely high frequencies in the population from Orkney Island (in Scotland) and in Biaka Pygmies from the Central African Republic (53–69%) *(data not shown)*.

The *delCFHR1* allele also displays considerable variation between racial/ethnic groups. Deletion homozygotes are most common in African Americans (16%), less common in Hispanics (6.8%) and least common in European Americans (4.7%) (Table 2). Analysis of the African samples in the HGDP shows the deletion to be frequent in many different African populations (Table 2). The high frequency of both the *CFH* Y402H allele and the *delCFHR1* allele in African populations suggests these alleles have an ancient origin. The high frequency of the deletion in African Americans may also help to explain the documented low frequency of late-onset AMD in this ethnic group, compared to the Caucasian population (48).

Discussion

Recently, our group and several others reported that polymorphisms in *CFH*, the gene encoding the principal soluble inhibitor of the alternative pathway of the complement cascade, are a major susceptibility factor for the development of AMD (9–12). A major "risk" *CFH* haplotype that confers increased susceptibility to AMD, and two "protective" haplotypes that confer a decreased risk, were identified (9).

In the process of serological screening, we noted that another member of the Factor H family, CFH-related protein 1 (CFHR1), was not detected in the serum of approximately 3% of individuals. We also noted that most individuals who lacked serum CFHR1 were homozygous for the normal nucleotide T at position 1277 of the *CFH* gene (which encodes the CFH$_{402Y}$ allele), and showed no evidence of AMD. Based upon these data, and the knowledge that the *CFH*-related genes share numerous regions of homology with *CFH*, we examined genetic variation in the RCA locus that includes *CFH* and the five *CFH*-related genes.

This analysis revealed a relatively common deletion encompassing the entire *CFHR1* and *CFHR3* genes. Although the precise breakpoints of the deletion were not determined, it includes DNA between the 3' end of exon 22 of *CFH* and the 5' end of exon 1 of the Complement Factor H-related 4 *(CFHR4)* gene. The breakpoints were further refined using PCR-specific primers and by chromosome walking (Figure 4 *and data not shown)*. Hughes and colleagues recently identified the same deletion (49). A recent study concluded that the functionally significant *CFH* S1191L/V1197A mutations in a patient with atypical hemolytic syndrome were caused by *de novo* gene conversion between *CFH* exon 22 and homologous sequences in exon 6 of *CFHR1* (50). Hughes and colleagues recently identified the same deletion (49). We suggest that such cases of gene conversion are more likely to occur when *CFH* and *CFHR1* are in closer proximity, such as they are on *CFHR1/CFHR3* deleted chromosomes.

Immunoblot analyses of sera from individuals homozygous for the *CFHR1/CFHR3* deletion confirmed the complete absence of the CFHR1 protein. This observation is consistent with a previous report showing the absence of CFHR1 in the sera of 4.4% of a healthy Caucasian population that was attributed to homozygosity for a null allele in *CFHR1* (51). Two more recent studies, which showed that large deletion variants in the human genome can account for a significant proportion of phenotypic variation, also identified a deletion in *CFHR1* (52,53). The *delCFHR1/CFHR3* mutation is most often associated with the previously identified *CFH* H4 haplotype that confers protection against AMD (9). This protection may be conferred by the absence of one or both of the CFHR proteins, or by altered function of the specific *CFH* haplotype(s) with which the deletion is in linkage disequilibrium. The homozygous *delCFHR1/CFHR3* is rarely present in individuals affected with AMD (observed in 1.1% of total cases; see Supplemental Table 6). Homozygous deletions were apparent in only three individuals with late-stage choroidal neovascularization (CNV), and in 11 subjects with clinical diagnoses of early AMD. Thus, the homozygous deletion appears to be associated with almost complete protection from late-stage AMD.

Both CFHR1 and CFHR3 contain SCRs that exhibit significant sequence homology with CFH SCRs 18–20 and 6–7, respectively (Figure 1). SCRs 6–7 contain binding sites for heparin, C-reactive protein, and several bacterial species. This domain also contains the Y402H variant of *CFH* that confers risk for AMD. The C-terminal domain of *CFH*, comprised of SCRs 18–20, has been characterized as mediating target recognition and self/non-self discrimination in the fluid phase. Function-blocking antibodies directed against the C-terminus (SCR20) block interaction of CFH with C3b, C3d, heparin, and binding to endothelial cells (54). Mutations in SCR20 have been shown to result in small vessel thrombosis (microangiopathy), thrombocytopenia, and acute renal failure that are characteristic of aHUS (55–57). Although the functional properties of CFHR1 and CFHR3 proteins are not fully defined, these proteins lack the complement regulatory activities ascribed to CFH SCRs 1–4, but retain some of the functions associated with other homologous CFH domains. For example, they bind C3b and C3d but not C3. Moreover, CFHR3 binds heparin (58), and its interaction with opsonized pneumococci can be inhibited by heparin (59). Since CFHR1 and CFHR3 both retain a C-terminal C3-binding domain, but lack the N-terminal domain responsible for inactivation of C3b, it is reasonable to suggest that they may function as competitive inhibitors of CFH, thus interfering with CFH binding and thereby having a negative effect on complement regulation. In such a model, the deletion of the *CFHR1* and *CFHR3* genes would lead to enhanced complement inhibitory activity by CFH and, therefore, reduced overall activation of the alternative pathway. Simply put, CFH may be more active in the absence of CFHR1 and CFHR3. In the context of AMD, this may be interpreted as protective if AMD susceptibility, as now believed, is influenced by dysregulation of complement activation via the alternative pathway.

Although the liver is responsible for the synthesis of most circulating CFH, our recent studies show that the eye is also a potential source of CFH, as well as a number of other complement components and complement regulators (13). *CFH*, but not *CFHR1* or *CFHR3*, transcripts can be detected by QPCR in the RPE/choroid complex and neural retina, the tissues most affected in AMD. The absence of *CFHR1* and *CFHR3* transcription suggests that functions ascribed to the respective proteins may be absent in these tissues or are mediated by systemic CFHRs produced in the liver.

The RCA locus displays a very complicated evolutionary and genetic history. The *CFH*-related genes were formed by gene duplication and several very large segmental duplications are present in this region. Both T1277C and the *CFHR1/CFHR3* deletions are frequent in multiple African populations as well as in European and Asian populations. This result is consistent with the proposition that these variants were selected for and have been maintained in the

population by balancing selection. Similarly, the *CFH* haplotype network analysis demonstrates a diverse set of *CFH* haplotypes, suggesting that there may be an advantage to maintaining population diversity in the regulation and specificity of the complement response.

The population genetic implications of the multiple protective and risk alleles in *CFH* region are consistent with an important role for the alternative pathway of the complement cascade in pathogen response. In other words, alleles with altered functionality may be maintained to protect against different pathogens. Robust complement activity directed against specific pathogens is desirable; however it may lead to a higher risk of inflammation-related, immune response disorders, particularly those with a late onset, which are not subject to selection. Similar diversity is found in other immune function genes such as in the MHC, the NK receptor genes, and immunoglobulin and T cell receptor genes.

Identification of a probable role for *CFH* in the pathogenetics of AMD has been a major step forward in unraveling the causes of this complex disorder. This finding, together with the subsequent discovery of AMD-associated variants in two related complement genes (*BF* and *C2*) (14,27), provide additional compelling evidence that the innate immune system and, more specifically, dysregulation of the alternative pathway of complement, plays a central role in the pathobiology of AMD. However, it is not yet apparent how protein isoforms encoded by major AMD risk-conferring gene variants mediate the molecular and cellular changes leading to AMD. One attractive scenario is that the inflammation triggered by exposure to infectious agents, or some other as yet undiscovered triggering event, in genetically susceptible individuals leads to the sustained activation of complement cascade, drusen formation and, eventually, the development of AMD. This scenario is consistent with the consequences of complement regulatory dysfunction in several rare kidney diseases, including membranoproliferative glomerulonephritis and atypical HUS (60,61).

In conclusion, we have demonstrated that there is a common and widespread deletion within the RCA locus that encompasses the *CFHR1* and *CFHR3* genes. The deletion is commonly found in many different populations in the world -- an indication of its ancient origin. The deletion allele is in high linkage disequilibrium with variants in *CFH*, and the *CFH* 1277T allele is found on the same haplotype as the deletion with 95% frequency. Those individuals who are homozygous for the *CFHR1/CFHR3* deletions and, therefore, do not express the respective proteins, are highly protected from developing AMD. Conversely, it appears that expression of the CFHR1 and CFHR3 proteins, in conjunction with other risk-conferring *CFH* isoforms, play permissive roles in the development of AMD and, perhaps, other age-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Web Resources

http://www.fluxus-engineering.com/sharenet.htm

http://genome.ucsc.edu

http://www.cephb.fr

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Abbreviations

AMD

   age-related macular degeneration

CFH

   complement Factor H

CFHR

   complement Factor H-related

RPE

   retinal pigmented epithelium

SSCP

   single strand conformation polymorphism

SDS-PAGE

   sodium dodecyl sulfate polyacrylamide gel electrophoresis

dNTP

   deoxyribonucleotide triophosphate

PBS

   phosphate buffered saline

HRP

   horseradish peroxidase

TBST

Tris-buffered saline Tween 20

QPCR

quantitative polymerase chain reaction

The Factor H Gene Family: Structure and Function
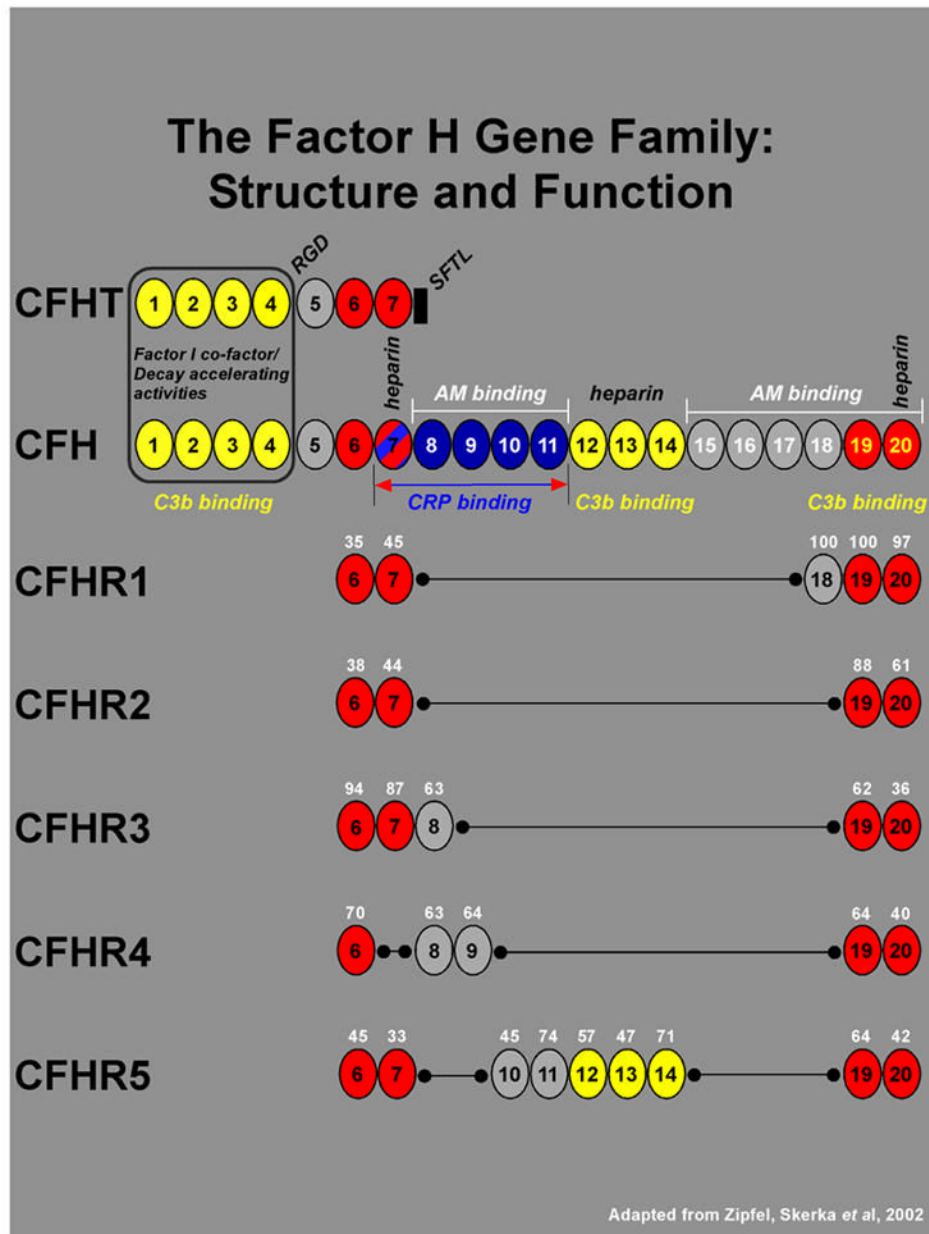
Adapted from Zipfel, Skerka *et al*, 2002

Figure 1.
The *CFH* gene contains 20 short consensus repeats (SCR), or complement control modules, each of which encodes a functional domain of ~60 amino acids. In addition to the full-length transcript, there is an alternatively-spliced, truncated gene product of *CFH* that has sequence identity with SCRs 1–7. An 8th exon spliced to SCR 7 in the truncated variant (black rectangle) encodes a stop codon, a unique untranslated region, and 4 C-terminal amino acids (SFTL). The full length and truncated forms of *CFH* transcripts are referred to as variants 1 and 2, and the respective encoded proteins are designated as isoforms a and b.
The 20 SCRs of *CFH* encode a number of functional domains. The complement Factor I co-factor and decay accelerating activities reside within SCRs 1–4 (boxed). Three C3b binding sites are associated with SCRs 1–4, 12–14, and 19–20 (shown in yellow). A RGD sequence in SCR 4 mediates cell adhesion through binding to complement receptor 3 (CR3), an αMβ2

integrin. A binding site for C-reactive protein (CRP), has been localized to the region spanning SCRs 7–11 (blue; in brackets), and a binding site for the *Streptococcus pneumoniae* surface protein β lies within a domain encompassing SCRs 8–11 (not shown). Low and high affinity adrenomedullin (AM) binding sites have been mapped to SCRs 8–11 and SCRs 15–20 respectively. Three heparin binding sites have been localized to SCRs 7, 12–14, and 20. Also located within SCR 20 is a sialic acid binding site (not shown).

The 5 related members of the *CFH* gene family are represented by 5 separate genes (gene symbols *CFHR1-5*) all of which reside within the regulators of complement activation (RCA) gene cluster on chromosome 1q32. Each of the 5 *CFH*-related genes contains a subset of the 20 *CFH* SCR domains (see Zipfel, Skerka *et al*, 2002 for review). The amino acid sequence homologies of *CFHR1-5* SCRs to their *CFH* counterparts are shown as a percentage in white numerals above each SCR. With the exception of *CFHR4* where SCR 7 is absent, all members of the *CFH* family contain two conserved regions corresponding to SCRs 6–7 and 19–20 of *CFH* (highlighted in red), and no members contain domains corresponding to SCRs 1–4 of *CFH*. Based upon their close structural relationships, it may be predicted that the five *CFH* -related family members also share functional similarities with *CFH*.
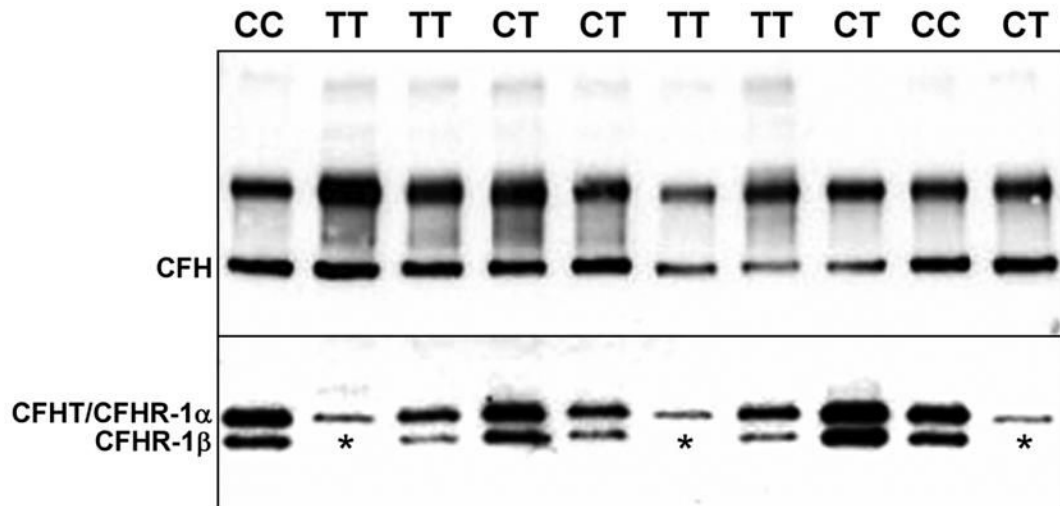
Figure 2.
A representative Western blot of serum proteins from 10 of 144 individuals analyzed by nonreducing SDS-PAGE and chemiluminescent immunoblotting. *(Panel A)* CFH is visualized as the faster migrating of the two bands (~150 kDa; identified as FH by co-migration with purified CFH; not shown) of two immunoreactive bands in the high molecular weight region of the gel/blot. The nature of the higher molecular weight CFH antibody-reactive band was not determined. The blot depicted was exposed to film for 3 seconds. *(Panel B)* CFHR1a, one of the most abundant proteins in serum, as well CFHR2 and the truncated form of CFH (CFHT/FHL-1) can be distinguished from CFH following separation of proteins in the lower molecular weight region of the gel/blot. The faster migrating band is CFHR-1β. No immunoreactive CFHR1 is present in the serum of three patients (lanes 2, 6 and 10), two unaffected patients possessing 1277TT genotypes (protective CFH H4 haplotype) and one patient with a 1277CT genotype, respectively. Subsequent SSCP analysis and direct DNA sequencing showed that both individuals both have a homozygous deletion of the *CFHR1* and *CFHR3* genes. The blot was exposed to film for 7 minutes.
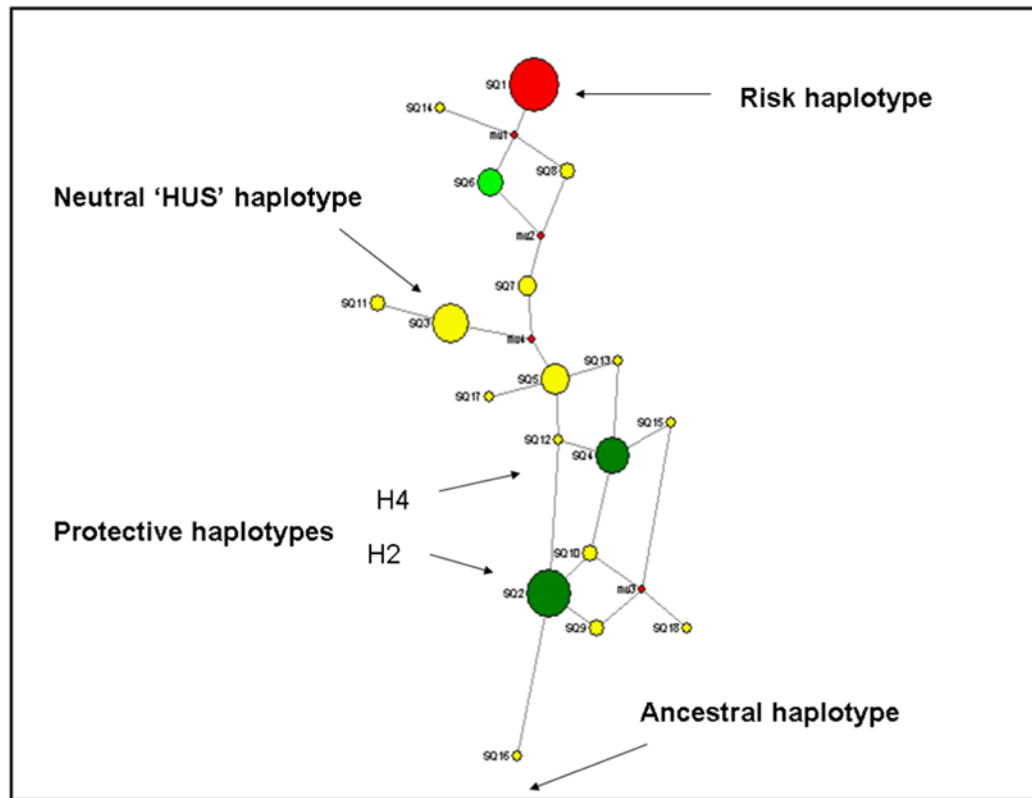
Figure 3.
A haplotype network resulting from the analysis of variants in the *CFH* locus is shown. The size of the sphere is proportional to the estimated frequency of the haplotype. Haplotypes adjacent to each other are predicted to arise from a single mutational step. The small spheres represent nodes that were not observed, but are predicted. The 402H haplotype is depicted, along with the two major protective haplotypes, and the haplotypes that are neutral for AMD risk.
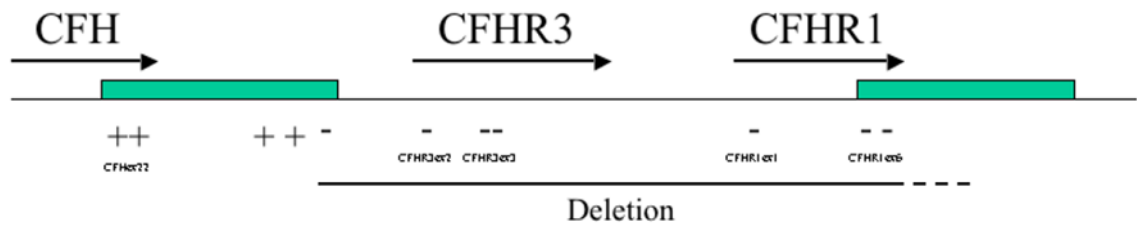
Figure 4.
A schematic map of that portion of the RCA cluster on 1q31 showing the relative positions of the *CFH*, *CFHR1* and *CFHR3* genes. The solid green boxes represent a segmental duplication of 28 kb that occurred within this locus. The primers employed to define the *CFHR1/CFHR3* deletion boundaries are shown in Supplemental Table 1; "+" indicates products that amplify in deletion homozygotes and "−" regions that fail to amplify in deletion homozygotes.

Table 1

CFHR1 Deletion Frequencies (Cases and Controls)

	Columbia		Iowa		Combined	
	Controls	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**
Count/[+++]	333	629	352	576	696	1213
Count del, del	24	6	18	7	42	14
SUM	357	635	370	583	738	1227
f $^+$, $^+$ & $^+$, del	0.933	0.9906	0.951	0.988	0.943	0.989
f del, del	0.067	0.0094	0.049	0.012	0.057	0.011
f$^+$	0.745	0.896	0.779	0.890	0.761	0.893
f del	0.255	0.104	0.221	0.110	0.239	0.107
X2/P	21.5/9.6E-07		10.5/0.0020		32.8/1.6E-09	
OR	0.158/0.61-0.39		0.248756/0.89-0.61		0.191/0.099-0.37	

[+] non-deleted allele; del, CFHR1/3 deletion; X2, Chi square value; P, p value; OR, odds ratio. The frequency of the deleted allele was calculated as the square root of the frequency of del/del homozygotes.

Table 2

Population Frequencies of *Y402H* and *delCFHR1/CFHR3*

CFH Y402H	TT	CT	CC	f[C]
HGDP Populations				
European	62	75	17	0.354
African	52	58	15	0.352
Middle Eastern	173	136	37	0.303
North African	15	14	1	0.267
Pacific	22	15	1	0.224
Asian	196	36	8	0.108
Southeast Asian	9	1	0	0.050
North American	90	5	0	0.026
South American	13	0	0	0.000
CFHR1/3 Deletion	**+/+, +/del**	**del/del**		**f[del]**
US Populations				
African American	292	55		0.159
Hispanic	248	18		0.068
European American	266	13		0.047
Chinese	92	2		0.022
HGDP Populations				
African	105	22		0.173
North African	24	5		0.172
Middle Eastern	180	31		0.147

The *CFH* Y402H variant was typed in the Human Genome Diversity Panel (HGDP) and the data separated by geographic regions. Individual genotypes were submitted to the HGDP database (http://www.cephb.fr). The *CFHR1/3* deletion was typed in populations of African Americans, Hispanics, Chinese, and European Americans as well a subset of HGDP.

Table 3

Ocular CFH, CFHT, CFHR1 and CFHR3 Gene Expression

	CFH	CFHT	CFHR1	CFHR3
Non-*delCFHR1/CFHR3*				
RPE/Choroid (n=6)	3.02E-01 +/− 1.08E-01	2.10E-01 +/− 8.52E-02	ND[1]	ND[2]
Retina (n=6)	1.56E-03 +/− 1.35E-03	6.98E-04 +/− 6.50E-04	1.69E-05 +/ − 1.15E-05[*]	ND[3]
delCFHR1/CFHR3				
RPE/Choroid (n=2)	2.84E-01 +/− 1.29E-01	1.16E-01 +/− 7.43E-02	ND	ND
Retina (n=2)	9.96E-04 +/− 4.41E-06	2.34E-04 +/− 7.85E-05	ND	ND
Liver	3.48E+00 +/− 7.89E-01	1.61E+00 +/− 3.12E-01	5.06E+00 +/− 5.77E-01	1.90E-01 +/ − 2.99E-02

Mean expression values of *CFH* and *CFH*-related genes were normalized to the geometric mean of two housekeeping genes (*GAPD, GPI*). For the non-del*CFHR1/CFHR3* individuals, the error values are designated as standard deviations. The error values for the *CFHR1/CFHR3* deletion homozygotes represent the range. ND (not detected above background levels). ND[1] (ND in 5/6 donors); ND[2,3] (ND in 6/6 donors);

[*] Detectable CFHR1 expression in 4/6 donors.