

A Hybrid *CFHR3-1* Gene Causes Familial C3 Glomerulopathy

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ABSTRACT

Controlled activation of the complement system, a key component of innate immunity, enables destruction of pathogens with minimal damage to host tissue. Complement factor H (CFH), which inhibits complement activation, and five CFH-related proteins (CFHR1–5) compose a family of structurally related molecules. Combined deletion of *CFHR3* and *CFHR1* is common and confers a protective effect in IgA nephropathy. Here, we report an autosomal dominant complement-mediated GN associated with abnormal increases in copy number across the *CFHR3* and *CFHR1* loci. In addition to normal copies of these genes, affected individuals carry a unique hybrid *CFHR3-1* gene. In addition to identifying an association between these genetic observations and complement-mediated kidney disease, these results provide insight into the protective role of the combined deletion of *CFHR3* and *CFHR1* in IgA nephropathy.

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Polymorphic variation within the *CFH-CFHR* gene locus influences susceptibility to diverse pathologies including age-related macular degeneration (AMD),¹ meningococcal sepsis,² and thrombotic and inflammatory kidney disease such as atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy, respectively.³ Recently, a common copy number polymorphism that results in the deletion of both *CFHR3* and *CFHR1* genes ($\Delta CFHR3-1$) has been associated with protection against the most common form of GN worldwide, IgA nephropathy, a pathology associated with complement deposition within the kidney.⁴ Although we have a detailed understanding of CFH biology, the biological role of the five CFHR proteins is unclear. It is likely that *CFHR5* has a role in the processing of complement within the kidney because it has

complement regulatory activity *in vitro*,⁵ it co-localizes with complement deposits within the kidney,⁶ and a *CFHR5* mutation is associated with familial complement-mediated GN.⁷ Although *CFHR1* and *CFHR3* have complement regulatory activity *in vitro*,^{8,9} both have been considered biologically redundant because their complete absence in healthy populations is common. For example, the $\Delta CFHR3-1$ homozygous deletion frequency is 4.7% in European-American and 16% in African-American populations.¹⁰ In fact, $\Delta CFHR3-1$ is actually associated with disease protection. $\Delta CFHR3-1$ is protective in both AMD^{10,11} and IgA nephropathy,⁴ pathologies associated with complement deposition in affected tissues. On the basis of a protective role for the $\Delta CFHR3-1$ in IgA nephropathy, we developed the hypothesis

that *CFHR1* and *CFHR3* impair complement processing within the kidney. This hypothesis would predict that an increase in *CFHR1* and *CFHR3* copy number would enhance susceptibility to complement-mediated kidney injury. Here, we report a novel *CFHR3-1* hybrid gene located on an allele that also contained intact copies of the *CFHR1* and *CFHR3* genes. The hybrid gene was detected exclusively in eight affected individuals among a large kindred with familial complement-mediated GN.

The clinical description of this family has previously been reported^{12,13} and the clinical progress of the affected individuals is described in Table 1. The kindred contains eight individuals with the condition over three generations (Figure 1). Renal biopsies were performed in six affected individuals. In the remaining two affected individuals, disease positivity

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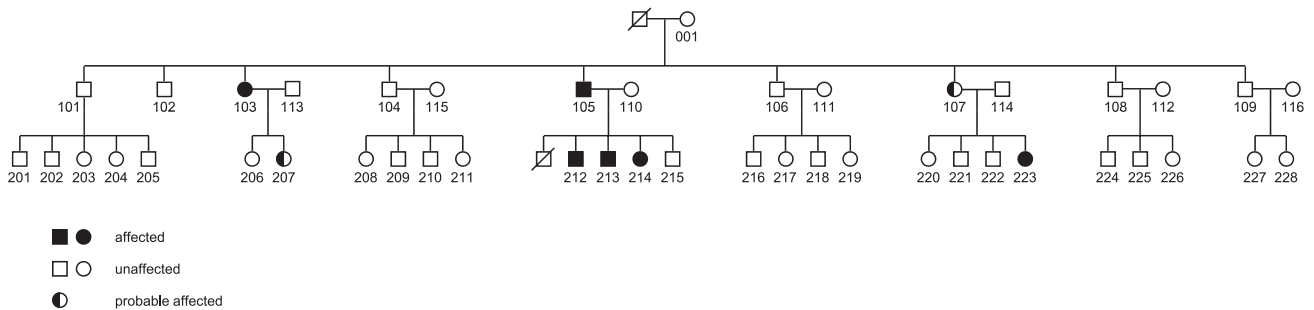
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Table 1. Updated clinical summaries of affected individuals

Pedigree No.	Presentation				End Stage Renal Failure	Renal Transplantation	Ocular Drusen
	Age (yr)	Proteinuria (g/24 h)	Hematuria	Biopsy Diagnosis			
103	25	>3	+++	MPGN	Yes	Yes	NA
105	51	3.06	++	MPGN	Yes	Yes	Absent
107	51	+++ ^a	+++	NA	No	—	NA
207	22	+++ ^a	+	NA	No	—	NA
212	28	3.77	+	MPGN	Yes	Yes	Absent
213	4	5.0	Negative	MPGN	Yes	Yes	Absent
214	21	3.13	+	MPGN	Yes	Yes	Absent
223	16	0.3	+++	MPGN	No	-	NA

MPGN, membranoproliferative GN; NA, not assessed.

^aQualitative proteinuria.**Figure 1.** Pedigree with familial C3 glomerulopathy. Affected individuals indicate those with C3 glomerulopathy confirmed on renal biopsy. Probable affected indicates those with significant proteinuria (>300 mg/24 h or at least 3+ on urinalysis) or hematuria (at least 3+ on two occasions). Unaffected individuals are healthy and do not have abnormal urinalysis.

was deduced from abnormal urinalysis (Figure 1). Renal biopsy showed a distinct pattern of glomerular inflammation termed membranoproliferative GN type III (MPGN type III). Evidence of glomerular immunostaining for C3 in the absence of Igs was present. These features indicated Ig-independent complement activation through the complement alternative pathway and are the defining features of C3 glomerulopathy, a term that includes MPGN type III, C3 GN, and dense deposit disease.³ AMD-like lesions may develop in C3 glomerulopathy but ophthalmologic assessment in individuals 105, 212, 213, and 214 did not reveal any evidence of ocular drusen. C3 glomerulopathy has been associated with genetic and acquired defects in complement regulation.³ No evidence of anti-CFH autoantibodies or C3 nephritic factors, acquired causes of C3 glomerulopathy, were detected in the affected individuals and plasma C3 and CFH levels were normal (Table 2). Previous linkage data indicated that the causative defect

was within a 22-cM region at chromosome 1q31–32 (maximum logarithm [base 10] of odds score = 3.86), which included the *CFH-CFHR* locus.¹³ Using Sanger sequencing, we did not identify any mutations in the *CFH* or the *CFHR* genes in the index case (pedigree number 105; Figure 1, data not shown). We next examined copy number variation (CNV) across the *CFH-CFHR* locus using multiplex ligation-dependent probe assay and TaqMan copy number assays (Supplemental Figure 1, A and B). Combined data from these approaches demonstrated the presence of three copies of two genomic segments within the *CFHR* locus. These segments included exons 1, 2, and 3 of *CFHR3* and exons 3, 4, 5, and 6 of *CFHR1* (Figure 2A). Abnormal rearrangement within the *CFH-CFHR* gene cluster can also impair the surface recognition domains of CFH and predispose individuals to familial aHUS.^{14,15} Importantly, in affected individuals, there was no evidence of structural mutations affecting the *CFH* gene, serum CFH levels were

normal, and anti-CFH antibodies were not detected.

We therefore hypothesized that a complex rearrangement that resulted in a *CFHR3-CFHR1* hybrid gene in addition to the normal diploid copies of both *CFHR3* and *CFHR1* genes had occurred, and that this was inherited in an autosomal dominant fashion in affected individuals. The region of interest (NCBI37/hg19: Chr1:196621008–196978800) containing the *CFHR* locus was successfully targeted using the Agilent SureSelect target enrichment system. Deep sequencing with coverage of >1000× was performed in one affected individual (pedigree number 214) and one related unaffected individual (pedigree number 104). Two partial gene duplication events were identified by comparing sequence coverage discrepancies between individuals; the first starting at least 10 kb upstream of *CFHR3*'s transcription start site and terminating in intron 3 of *CFHR3*, the second starting in intron 1 of *CFHR1* and terminating at least 13 kb downstream of *CFHR1* (Supplemental

Table 2. Complement profile

Pedigree No.	Status	C3	C4	CH100	AP100	CFH ^a	CFI ^a	C3NeF	Anti-CFH
Normal range		0.7–1.7 g/L	0.16–0.54 g/L	50%–150% NHP	50%–150% NHP	% NHP	% NHP	n/a	AEU
103	Affected	0.93	0.39	158	109	102	152	ND	ND
105	Affected	0.84	0.29	155	39	77	87	ND	ND
212	Affected	1.16	0.54	126	108	119	123	ND	ND
213	Affected	1.12	0.40	172	32	84	96	ND	ND
214	Affected	0.79	0.26	145	64	86	103	ND	ND
215	Unaffected	1.12	0.23	155	74	77	96	ND	ND

NHP, normal human pool; C3NeF, C3 nephritic factor; AEU, arbitrary ELISA units; CFI, complement factor I; ND, not detected.

^aNo validated reference range exists for CFH and CFI and results are thus expressed as the percentage of NHP. C3NeF was detected using a commercial assay (The Binding Site, UK).

Figure 1C). Analysis of the sequencing which compared atypical mapping of paired-end reads between these individuals revealed two genomic breakpoints (NCBI37/hg19:chr1:196754062 at *CFHR3* intron 3 and NCBI37/hg19:chr1:196790079 at *CFHR1* intron 1) present only in the affected individual (Supplemental Figures 2 and 3).

To confirm the presence of these genomic breakpoints, primers located within the duplicated regions were used to generate a 6-kb amplicon. As expected, it was possible to generate this amplicon using genomic DNA from affected individuals only. Amplicon sequencing confirmed the genomic breakpoints identified using the paired-end mapping analysis. These data indicated a *CFHR3-CFHR1* hybrid gene comprising exons 1, 2, and 3 from *CFHR3* and exons 2, 3, 4, 5, and 6 from *CFHR1* (Figure 2A). This hybrid gene was present on a *CFH-CFHR* allele that contained intact copies of the *CFHR1* and *CFHR3* genes. We next designed a genomic PCR that would amplify this breakpoint (757-bp amplicon) in addition to normal genomic sequence (366-bp amplicon). The abnormal amplicon was present in all affected members of the kindred but not in unaffected individuals (Supplemental Figure 4) or 450 Caucasian control samples (data not shown).

Analysis of the predicted hybrid protein cDNA sequence indicated that splicing from *CFHR3* exon 3 to *CFHR1* exon 2 would generate a novel protein containing 454 amino acids. The protein would comprise leader peptide sequence and the initial two protein domains, termed short consensus repeat domains, of *CFHR3* (derived from *CFHR3* exons 1, 2, and 3)

and all five short consensus repeat domains of *CFHR1* (derived from *CFHR1* exons 2, 3, 4, 5, and 6; Figure 2A). Using a polyclonal anti-human CFH antibody we were able to demonstrate the presence of two aberrant protein bands of similar molecular weight in affected but not unaffected sera (Figure 2B). Two *CFHR1* protein bands of similar molecular mass (37-kD *CFHR1* α and 43-kD *CFHR1* β) are seen in sera and we postulated that the two aberrant protein bands represented differentially glycosylated isoforms of the *CFHR3-1* hybrid protein (Figure 2B). Using heparin-purified sera from affected individuals, we were able to isolate and analyze the aberrant proteins by peptide mass fingerprinting (Supplemental Figure 5). This analysis identified seven peptides within the predicted hybrid protein sequence (Supplemental Figure 5), including a unique peptide sequence that spanned the junction between *CFHR3* protein sequence and *CFHR1* protein sequence (VTTFCDFFPK) (Supplemental Figure 5).

It is important to note that linkage to the disease locus was reported in this family a decade ago¹³ and exon sequencing of the *CFH-CFHR* locus did not detect the structural variation. Paired-end mapping analysis and copy number assays across the locus successfully identified the hybrid *CFHR3-1* gene. Hence, target enrichment followed by paired-end mapping analysis is an efficient method of detecting rearrangements and CNV in linkage peaks and this approach can be applied on a genome-wide level.^{16,17}

The *CFHR3-1* hybrid gene is a unique cause of C3 glomerulopathy. The association is robust because we

were able to demonstrate complete segregation with disease in a large kindred. It is likely that this hybrid gene arose as a result of an abnormal crossover event during meiosis. Frequent interspersed repeat elements within the *CFH-CFHR* locus most likely predispose to such events.¹⁸ The association between an abnormal *CFHR3-1* hybrid gene on an allele with intact copies of both *CFHR3* and *CFHR1* in familial C3 glomerulopathy, together with the observations that Δ *CFHR3-1* is common among healthy individuals and protective in IgA nephropathy and AMD, suggested that the *CFHR3-1* protein causes C3 glomerulopathy through a dominant mechanism. However, further studies are needed to demonstrate causation in this family. In a previous study, we identified a heterozygous internal duplication within *CFHR5* in familial C3 glomerulopathy.⁷ Whether this represented a loss of function or dominant negative change remained uncertain because the consequences of complete *CFHR5* deficiency, unlike complete deficiency of *CFHR1* and *CFHR3*, were unknown. We speculate that the *CFHR3-1* and *CFHR5* mutant proteins mediate abnormal accumulation of C3 within the kidney through a common dominant mechanism most likely interfering with the C3 regulation by CFH and *CFHR5*. It may be the case that normal *CFHR3* and *CFHR1* proteins also interfere with this process, albeit to a lesser degree; consequently, the absence of these proteins, afforded by the Δ *CFHR3-1* polymorphism, results in amelioration of complement-mediated renal injury in IgA nephropathy.

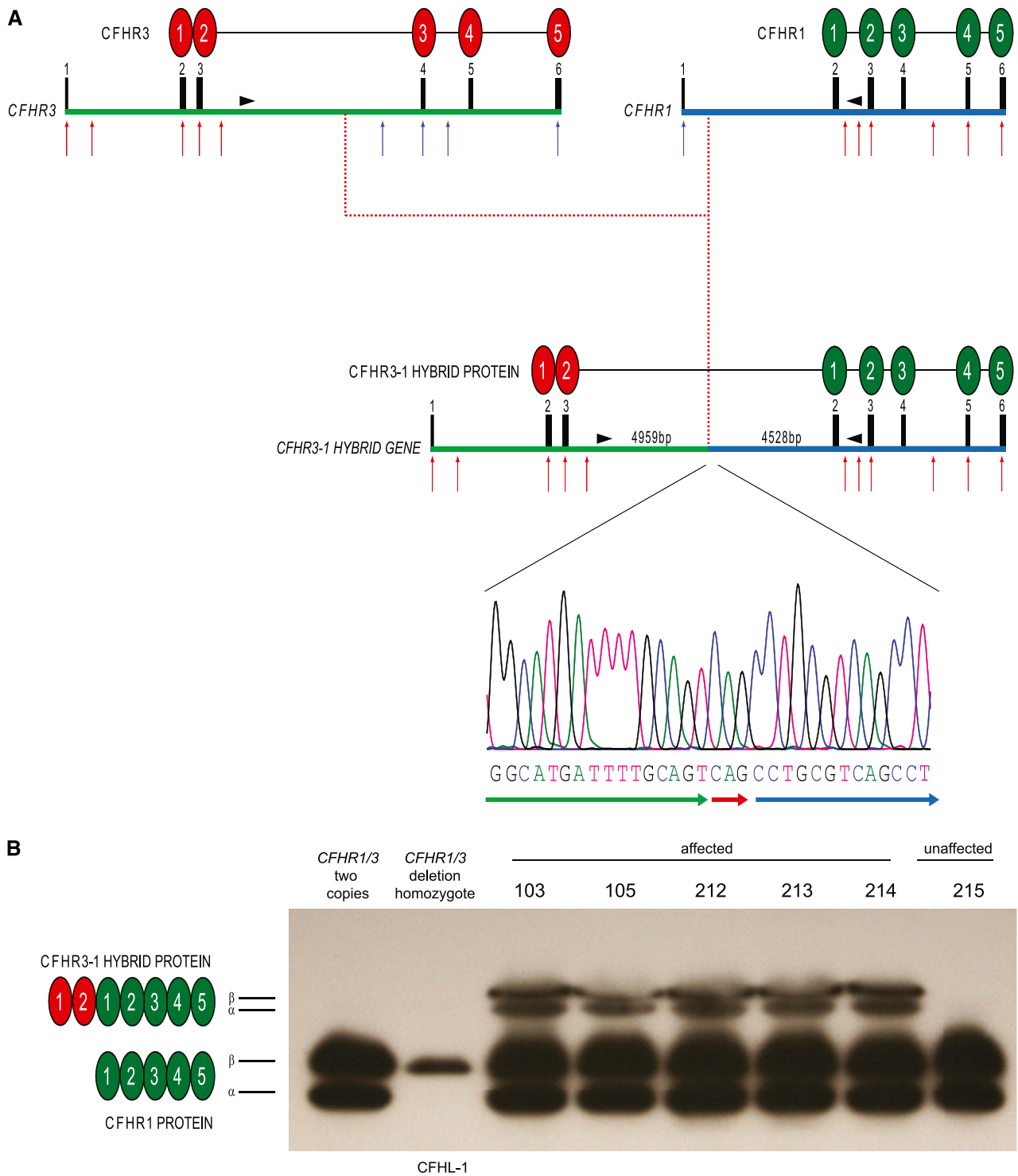


Figure 2. The *CFHR3-1* hybrid gene structure, genomic breakpoint, and protein. (A) Schematic representation of the *CFHR3-1* hybrid gene and protein. Red arrows indicate sites of probes giving three copy signals, whereas blue arrows depict probes giving two copy signals using copy number assays. Exons and protein domains are numbered. The intergenic distance between *CFHR3* and *CFHR1* is 24 kb. The genomic breakpoint (4959 bp into *CFHR3* intron 3) was identified using paired-end mapping analysis of next-generation sequence and long-range genomic PCR and amplicon sequencing. The breakpoint sequence contained a CAG triplet not identified in the introns of either gene (red arrow). (B) Serum Western blot using polyclonal anti-CFH antibody demonstrated the presence of two additional protein bands of similar molecular mass only in sera from affected individuals. The two bands represent differentially glycosylated isoforms (designated α and β) comparable with that seen with *CFHR1*. CFHL-1, CFH-like protein 1, a protein derived from alternative splicing of the *CFH* gene that is visible on this gel only in the absence of *CFHR1* protein (lane 2).

CONCISE METHODS

Sequencing

Exon sequencing of *CFH* and *CFHR1–5* genes was performed in two affected individuals (numbers 105 and 213). Amplicons that targeted the exons and at least 50 bp of contiguous intronic sequence were amplified by PCR using primers designed using Primer3 software. Sequencing reactions were performed using BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Both PCR and sequencing reaction products were purified using Edge Biosystems Gel Filtration plates (Edge Biosystems, Gaithersburg, MD). The sequencing was run on a 3730 DNA Analyzer and analyzed using Sequencher DNA sequencing analysis software (Gene Codes Corp, Ann Arbor, MI).

Multiplex Ligation-Dependent Probe Amplification

Probes used targeted *CFH* exons 1, 2, 3, 4, 6, 11, 12, 13, 18, and 23; *CFHR3* exons 1, 2, 3, 4, and 6; *CFHR1* exons 1, 3, 5, and 6; *CFHR2* exons 1, 2, 3, and 4; and *CFHR5* exons 1, 2, and 3 (MLPA P236-A1; MRC Holland, www.mlpa.com). Genomic DNA was processed according to the manufacturer's instructions. Assay readings were validated using control samples that included individuals with known heterozygous or homozygous polymorphic deletion of the *CFHR1* and *CFHR3* genes. Data were depicted as mean \pm SD.

CNV with Real-Time PCR

TaqMan probes (Applied Biosystems) were used to further dissect CNV within the *CFHR3* and *CFHR1* genes. Genomic DNA was processed according to manufacturer's instructions. Assay readings were normalized to control samples and values represent mean \pm SD. All probes were validated using genomic DNA from controls with either heterozygous or homozygous polymorphic deletion of the *CFHR1* and *CFHR3* genes.

In-Solution Target Enrichment of the *CFHR* Locus and Next-Generation Sequencing

Extracted DNA from affected individual 214 and unaffected individual 104 were controlled using fluorescent dosage with picogreen as well as Nanodrop analysis. Illumina libraries were

constructed according to the manufacturer's SureSelect protocol, which involves replacement of one of the Illumina paired-end adapters with the SureSelect precapture adapter and precapture PCR. Library construction was carried out using the automated SPRI-TE system (Beckman Coulter Genomics). Libraries were controlled on the Agilent Bioanalyzer. In parallel, baits were designed on the *CFHR* locus defined as chr1:196621008–196978804 using the online Agilent eArray design tool. Default design parameters were used to generate a total of 2179 baits of 120-bp length, covering 150 kb of the 358-kb region of interest, essentially avoiding nonunique locations such as long interspersed nuclear element repeats and long terminal repeats with the default limited 20-bp allowance of bait overlap with nonunique sequence. This represents around 4% of bait space provided for custom bait libraries by the manufacturer. Expected actual extent covered by captured fragments is in general several tens of kilobases higher because the method also captures fragments having only partial overlaps with the 120-bp reads. The design was visually checked on the UCSC genome browser (University of California, Santa Cruz, California). Baits were ordered from the manufacturer; upon reception, recommended quantities of baits were mixed with denatured Illumina libraries and hybridization was carried out over 24 hours. After capture, noncaptured DNA was washed away, the hybridized molecules were eluted, and postcapture PCR was performed integrating the Illumina 3' index adapters so as to tag the libraries with DNA barcodes. Library quality control and dosage was carried out so as to pool an equivalent number of molecules from the two libraries for sequencing in a single lane. Paired-end 2 \times 50-bp sequencing was performed on the Illumina Genome Analyzer IIx, generating 22M read pairs for the affected and 18M read pairs for the unaffected individuals.

Breakpoint Analyses

Sequences were mapped on the human genome by the manufacturer's Eland program, comprised in the Illumina CASAVA 1.8 software suite. The mapped reads were realigned using the SRMA 0.15 assembler. Resulting sequence coverage was examined using Integrative Genomics Viewer software. Subsequently, atypical pairs were isolated using a BAMtools

0.9 custom filter, specifying insert sizes >1 kb. These atypical pairs were provided as input to SVDetect 0.7. SVDetect was instructed to compare the two libraries so as to isolate locations with atypical read pairs present in the affected sequence set and absent from the unaffected sequence set. Finally, Circos 0.55 was configured to project the output from SVDetect onto a circular *CFHR* locus representation (Supplemental Figure 3A). The output from SVDetect was also uploaded onto the Santa Cruz UCSC genome browser so as to compare putative breakpoint locations against known transcripts (Supplemental Figure 3B).

PCR

We designed a genomic PCR that would generate a single 366-bp amplicon in individuals with at least one copy of the *CFHR3* and *CFHR1* genes using the following primers: forward, 5'CCTTGTGACTTTCCATCTCG3'; and reverse, 5'AGGAAACCCATCTCATGTGC3', which targets GCACATGAGATGGGTTTCCT in *CFHR3* intron 3–4. In the presence of the *CFHR3–1* hybrid gene, an additional 757-bp amplicon is generated using the same forward primer: 5'CCTTGTGACTTTCCATCTCG3' and the reverse primer 5'GGTGGCTTATGCGTCAA3', which targets TTGCAGGCATAAGCCACC in the forward strand of *CFHR1* intron 1–2. PCR was performed using Qiagen HotStar Taq DNA polymerase with 10 ng of genomic DNA according to the manufacturer's protocol. Both reactions were multiplexed in the same reaction. Affected individuals had both a 366-bp and a 757-bp amplicon. In contrast, only the 366-bp control amplicon was seen in all unaffected individuals examined.

Western Blots

Western blot for CFHR proteins was performed using heparin-purified sera from healthy controls and an affected individual (Supplemental Figure 3A). Samples were enriched for CFHR proteins using heparin affinity chromatography and immunoprecipitation. Samples were incubated with heparin-coated sepharose beads (GE Healthcare). After centrifugation and washing, bound proteins were eluted with 500 mM NaCl, 20 mM TRIS-HCl, pH 7.5. Eluted proteins were immunoprecipitated with a mouse monoclonal anti-human CFHR1 antibody (a gift from Dr. Claire Harris, Cardiff University, Wales), which recognizes an epitope common to CFHR1, CFHR2, and

CFHR5, and run on standard 12% SDS PAGE under nonreducing conditions and probed with a goat polyclonal anti-human CFH antibody (Quidel).

Peptide Mass Fingerprinting

After electrophoresis, a replicate gel was stained with Coomassie blue and the aberrant bands were manually excised and the gel fragment subjected to peptide mass fingerprinting. Protein samples were reduced and alkylated with iodoacetamide and then digested with trypsin. Peptide mass determination was performed using a Bruker Autoflex III MALDI TOF/TOF instrument by Alphalyse (www.alphalyse.com).

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DISCLOSURES

None.

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