

Deletion of Complement Factor H–Related Genes *CFHR1* and *CFHR3* Is Associated with Atypical Hemolytic Uremic Syndrome

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Atypical hemolytic uremic syndrome (aHUS) is associated with defective complement regulation. Disease-associated mutations have been described in the genes encoding the complement regulators complement factor H, membrane cofactor protein, factor B, and factor I. In this study, we show in two independent cohorts of aHUS patients that deletion of two closely related genes, complement factor H–related 1 (*CFHR1*) and complement factor H–related 3 (*CFHR3*), increases the risk of aHUS. Amplification analysis and sequencing of genomic DNA of three affected individuals revealed a chromosomal deletion of ~84 kb in the RCA gene cluster, resulting in loss of the genes coding for *CFHR1* and *CFHR3*, but leaving the genomic structure of factor H intact. The *CFHR1* and *CFHR3* genes are flanked by long homologous repeats with long interspersed nuclear elements (retrotransposons) and we suggest that nonallelic homologous recombination between these repeats results in the loss of the two genes. Impaired protection of erythrocytes from complement activation is observed in the serum of aHUS patients deficient in *CFHR1* and *CFHR3*, thus suggesting a regulatory role for *CFHR1* and *CFHR3* in complement activation. The identification of *CFHR1/CFHR3* deficiency in aHUS patients may lead to the design of new diagnostic approaches, such as enhanced testing for these genes.

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Introduction

Atypical hemolytic uremic syndrome (aHUS) is characterized by a triad consisting of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure in the absence of a preceding diarrheal illness. aHUS can be either sporadic or familial. Defective complement regulation occurs in both sporadic and familial aHUS. Disease-associated mutations have been described for the genes encoding the complement regulators complement factor H (CFH), membrane cofactor protein, factor I, and factor B [1–4]. In addition, autoantibodies to factor H have been reported in aHUS patients [5]. Recently, we showed in a family with aHUS that nonallelic homologous recombination [6] results in the formation of a hybrid gene derived from exons 1–21 of *CFH* and exons 5–6 of complement factor H–related 1 (*CFHR1*) [7]. The protein product of this hybrid gene is identical to the aHUS-associated CFH mutant S1191L/V1197A, which arises through gene conversion [8]. *CFH* and the genes encoding the five complement factor H–related proteins (*CFHR1–CFHR5*) reside in a centromeric 355-kb segment on Chromosome 1. Sequence analysis of this region provides evidence for multiple independent large genomic duplications, also known as low-copy repeats, resulting in a high degree of sequence identity between *CFH* and *CFHR1–CFHR5* [9, 10]. The secreted protein products of these genes are related in structure, as they are composed of repetitive units (~60 amino acids) named short consensus repeats (SCRs) [11]. In

this study, we describe a novel form of nonallelic homologous recombination that results in the deletion of *CFHR1* and *CFHR3*, but leaves *CFH* intact. This deletion is associated with an increased risk of aHUS.

Results/Discussion

Two cohorts of patients with aHUS have been studied, one from Jena, Germany and one from Newcastle, United Kingdom. For the Jena cohort of 121 aHUS patients, we used Western blotting to determine the absence of *CFHR1* and *CFHR3* in serum, as demonstrated for three patients in Figure 1A–1C. Complete absence of both *CFHR1* and *CFHR3*

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Abbreviations: aHUS, atypical hemolytic uremic syndrome; CFH, complement factor H; CFHR, complement factor H–related; HUS, hemolytic uremic syndrome; MLPA, multiplex ligation–dependent probe amplification; SCR, short consensus repeats

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Author Summary

Hemolytic uremic syndrome (HUS) is a severe kidney disease, which is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. The nondiarrhea-associated form, also known as atypical HUS (aHUS), is rare, sometimes familial, often recurrent, and has a poor outcome. Several studies have shown that aHUS is associated with mutations in genes coding for complement regulators, which leads to defective regulation of complement activation, particularly at cell surfaces. We report a novel susceptibility factor for aHUS in the form of a chromosomal deletion of a large (~84 kb) genomic fragment in the regulators of complement activation gene cluster at Chromosome 1q32. This deletion is a result of nonallelic homologous recombination and leads to the loss of two genes, *CFHR1* and *CFHR3*, which encode factor H-related proteins 1 and 3, respectively. We recommend diagnostic screening of aHUS patients for these susceptibility factors.

but presence of factor H, factor H-like protein 1, CFHR2, and CFHR4A was detected in 19 aHUS patients (16%) compared to two out of 100 control participants ($\chi^2 = 10.4$, $p = 0.0012$, odds ratio = 8.5). All 19 patients showed normal factor H serum levels. In three of these 19 patients, DNA analysis confirmed that the deficiency was caused by a homozygous genomic deletion. The *CFH* genes were normal, as determined by sequence analysis. Specific primers were designed which span the 113-kb region from the 3' exons of *CFH* to *CFHR4* (Figure 2A). Failure of primers R2–R6 to amplify DNA of these patients is explained by a 84-kb deletion of a genomic fragment that includes *CFHR3* and *CFHR1* and is located downstream of *CFH* and upstream of

CFHR4. This deletion is flanked by two duplicated segments, B and B', which are 28,638 bp and 28,714 bp in length, respectively. B includes exons 21, 22, and 23 of *CFH* and is located 5' of *CFHR3*. B' includes exons 3, 4, and 5 of *CFHR1* and is located ~60 kb further downstream. Both segments have the same orientation, harbor several truncated long interspersed nuclear elements, and their sequence identity is >98 % [12]. The position of the deletion was mapped by amplifying regions of sequence variation between the duplicated segments. Forward and reverse primers specific for B and B', respectively, generated a 9.2-kb product from aHUS patients' DNA, but not from control DNA. Sequence analysis allowed the identification of nucleotides from either B or B' (Figure 2B), thus demonstrating fusion of B and B' as a result of nonallelic homologous recombination. This was confirmed by amplification of the fused segment BB' using primers that also generate amplification products from segment B and segment B' of control DNA. Sequence analysis of a divergent region in B and B' confirmed that amplification of DNA from the patient generated one product with a single sequence (Figure 2C, upper panel), while the control DNA generated two products with divergent sequences (Figure 2C, middle panel), of which one is derived from segment B and one from segment B' (Figure 2C, lower panel). These data demonstrate fusion of segments B and B' in the patient's DNA, and confirm that this patient is homozygous for the deletion (Figure 3A). The same results were obtained for the other patients (unpublished data). All three patients had identical breakpoints, which were mapped to a 1.9-kb region of perfect homology within or directly preceding a LIMA2 element (Figure 3B).

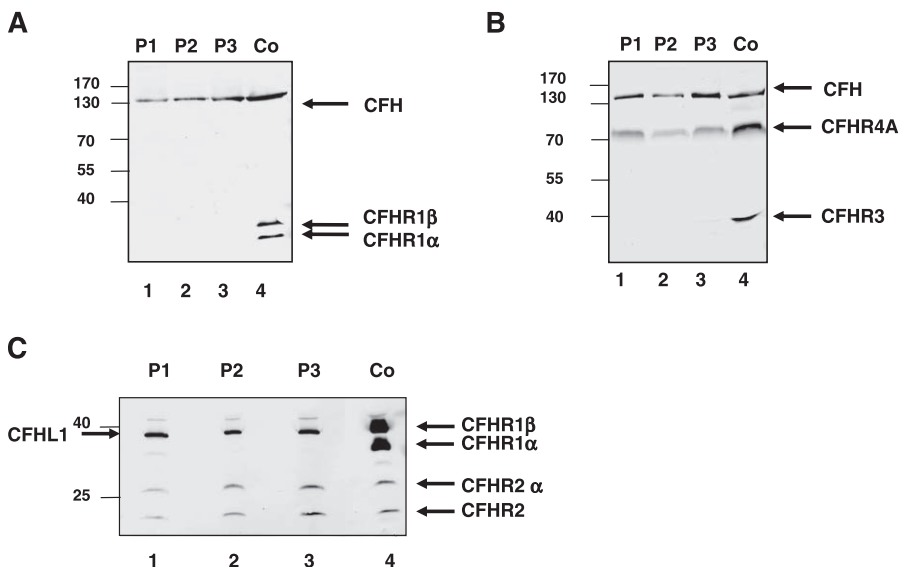


Figure 1. Deficiency of CFHR1 and CFHR3 in HUS

Western blotting of serum from three HUS patients (lanes 1–3) and a control subject (lane 4).

(A) Using a monoclonal antibody that reacts with the C terminus of CFHR1, complete deficiency of CFHR1 is seen in the three HUS patients. The normal control shows the two differently glycosylated forms of CFHR1 (CFHR1 α and CFHR1 β , respectively).

(B) Using CFHR3 antiserum, complete deficiency of CFHR3 is seen in three HUS patients, but CFHR3 is detected in the control subject. CFHR4A is present in all samples.

(C) The sera of the three HUS patients contain factor H-like protein 1 and the glycosylated and nonglycosylated forms of CFHR2 (CFHR2 α and CFHR2, respectively).

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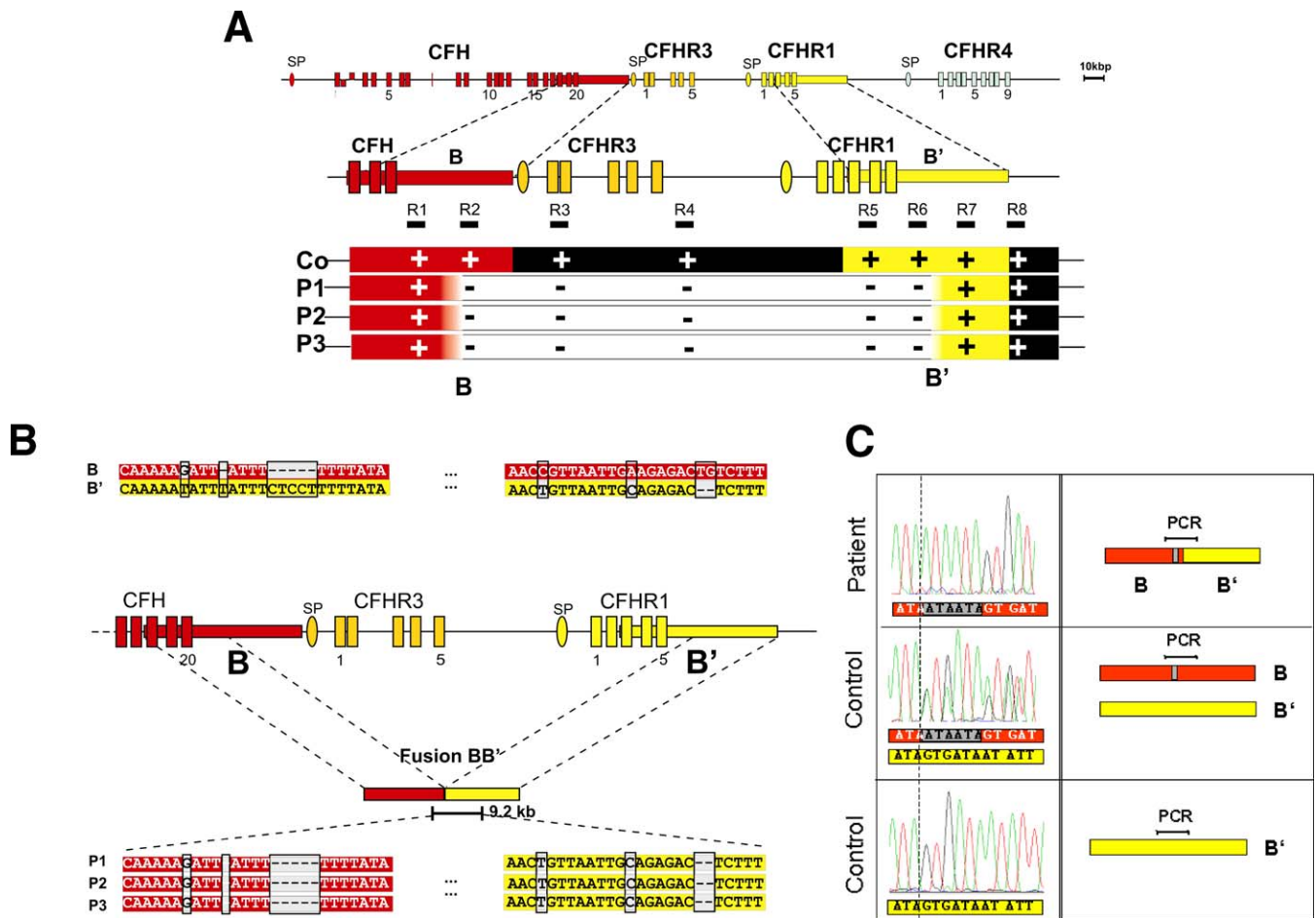


Figure 2. Map of the Human Factor H Gene Cluster

(A) The 20 SCRs of *CFH*, the five SCRs of *CFHR3*, the five SCRs of *CFHR1*, and the nine SCRs of *CFHR4* are indicated by vertical bars. The position of the two duplicated homologous segments (B and B') and that of the eight primer pairs (R1–R8) is shown. Failure (–) to amplify DNA downstream of *CFH* in the three HUS patients reveals a deletion of a genomic ~84-kb fragment.

(B) Sequence comparison of segment B (red) and segment B' (yellow) reveals blocks with sequence diversity. Fusion of the homologous segments and localization of the breakpoints in three *CFHR1/CFHR3* deficient HUS patients are indicated. Fusion of segments B and B' is demonstrated by amplification of a 9.2-kb PCR fragment using a forward primer specific for segment B and a reverse primer specific for segment B'. For all three patients, sequences of block 1 are derived from segment B (red) and sequences of block 2 are derived from segment B' (yellow).

(C) Similar PCR products were generated with primers common for segment B and segment B'. Amplification of both segments is confirmed by sequence analysis. Sequence of PCR products from the patient is identical to segment B of a control DNA (upper panel). Sequence of PCR products of a control person is identical to segment B and B' (middle panel). Identification of sequence B' of the control person (lower panel). Sequence differences between segment B and segment B' are indicated (grey bar).

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The Newcastle cohort of 66 aHUS patients was investigated using multiplex ligation-dependent probe amplification (MLPA) [13] to measure copy number of *CFHR1* exons 2 and 3 (Table 1). The data show that deletion of *CFHR1* is strongly associated with aHUS. In the aHUS group, 28% of the patients had this deletion, compared to 6% of the control group ($\chi^2 = 33.2$, $p = 1.0 \times 10^{-8}$, odds ratio = 6.3). The following copy numbers of *CFHR1* were found in aHUS patients: zero copies, 10%; one copy, 35%; two copies, 55%. In control subjects, the copy numbers were: zero copies, 2%; one copy, 9%; and two copies 89% ($\chi^2 = 28.7$, $p = 5.9 \times 10^{-7}$). The allele frequency of *CFHR1* deletion was 30% in those patients known to carry a mutation and 27% in those without a mutation ($\chi^2 = 0.16$, $p = 0.69$).

The functional effect of complete deficiency of *CFHR1* and *CFHR3* proteins was investigated using a modified version of

a hemolytic assay with factor H-depleted plasma and sheep erythrocytes [14, 15]. Addition of heat-inactivated serum from the three patients caused increased erythrocyte lysis (Figure 4). In contrast, serum derived from a healthy individual showed dose-dependent protection. These data show that *CFHR1/CFHR3*-deficient plasma has reduced protective activity and suggest that the absence of *CFHR1* and/or *CFHR3* contributes to the defective regulation of complement activation on cell and tissue surfaces. This is underlined by the fact that these patients have normal serum levels of factor H and factor I. Similarly, we previously showed that mutant factor H derived from HUS patients displayed reduced cell binding and protection activities [16].

In this study we report that hetero- and homozygous deletion of *CFHR1* and *CFHR3* through nonallelic homologous recombination events downstream of *CFH* is associated

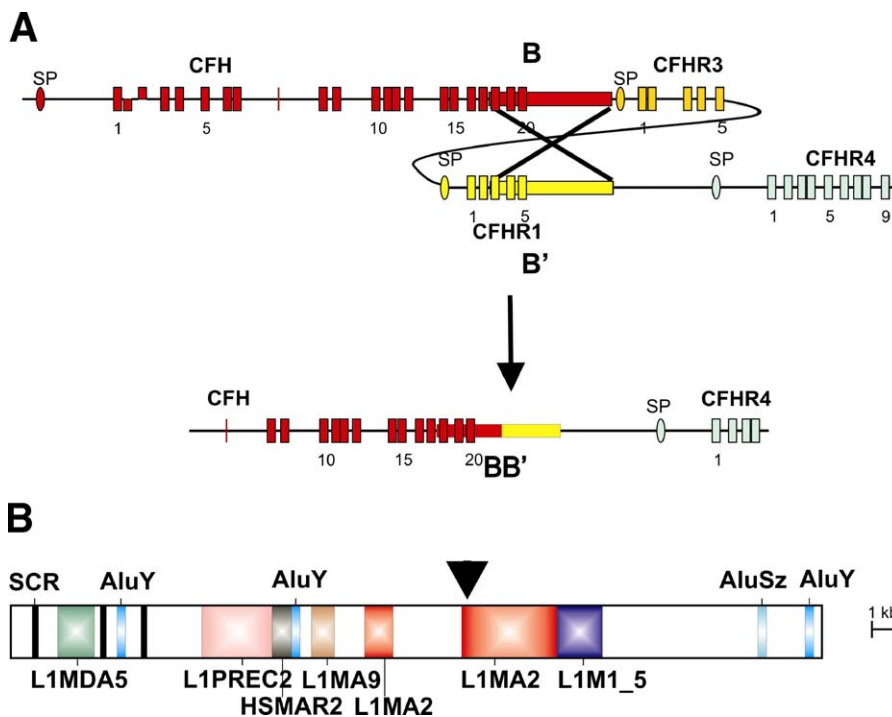


Figure 3. Chromosomal Rearrangement of Segments B and B'

(A) Homologous recombination between segments B and B' in the RCA gene cluster results in deletion of the genes coding for CFHR1 and CFHR3 and fusion of segments B and B' as described in the text.

(B) The B and B' segments in the factor H gene cluster are characterized by high sequence identity (98%) and include several truncated long interspersed nuclear elements (retrotransposons). The breakpoint identified in all three patients is indicated by the arrow.

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with an increased risk of aHUS. aHUS patients with deficiency of CFHR1/CFHR3 are characterized by a relatively young age (1–21 y) at disease onset. Previously, we showed that deletion of CFHR1 and CFHR3 reduces the risk of age-related macular degeneration [17]. It is fascinating that the same polymorphic variant is associated with opposite effects: an increased risk for HUS and a decreased risk for age-related macular degeneration. How could this deletion be acting? One possibility is that the absence or presence of either CFHR1 and/or CFHR3 has a disease-modifying action. Although each of the two proteins alone lacks cofactor and decay-accelerating activity, CFHR3 has a cofactor-enhancing activity [18]. In addition, both CFHR1 and CFHR3 bind C3b and heparin, thus suggesting a regulatory function in C3b processing [18].

Another possibility is that the deletion is in linkage disequilibrium with other susceptibility alleles in *CFH* or

that it may affect *CFH* transcription. Understanding the functional effect of this disease-modifying deletion will help to extend our understanding of the role of complement in the pathogenesis of both aHUS and age-related macular degeneration.

Materials and Methods

Participants. *Newcastle.* Sixty-six patients from Newcastle with a clinical diagnosis of aHUS were included in this study. Within this group, 15 were known to carry a *CFH* mutation, two carried a membrane cofactor protein mutation, and three carried a factor I mutation. Screening with MLPA for exons 22 and 23 of *CFH* showed that none of these patients carried a hybrid *CFH/CFHR1* gene. The allele frequency of *CFHR1* deletion in the aHUS patients was compared to 119 samples of human control DNA from a randomly selected population (healthy blood donors) obtained from the European Collection of Cell Cultures (<http://www.ecacc.org.uk>). The study was approved by the Northern and Yorkshire Multi-Centre Research Ethics Committee.

Table 1. Frequency of *CFHR1* Deletion and Copy Number of *CFHR1* Exons 2 and 3 in aHUS Patients and Controls (Newcastle Cohort)

Study Participants	<i>CFHR1</i> Allele Frequency ^a		<i>CFHR1</i> Copy Number ^b		
	Deleted	Present	0	1	2
aHUS patients	37	95	7	23	36
Controls	15	223	2	11	106

^a $\chi^2 = 33.2$, $p = 1.0 \times 10^{-8}$

^b $\chi^2 = 28.7$, $p = 5.9 \times 10^{-7}$

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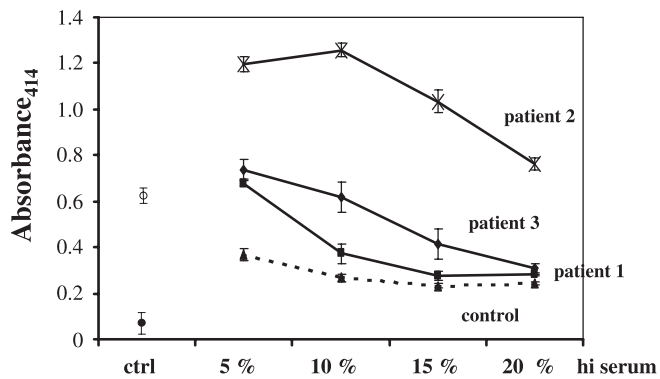


Figure 4. Defective Protective Activity with CFHR1/CFHR3 Deficient Plasma

A novel type of hemolytic assay was used to analyze hemolytic activity of CFHR1/CFHR3 deficient human serum. Sheep erythrocytes were incubated with factor H-depleted human plasma. Sheep erythrocytes generally act as nonactivators of complement in human plasma. However, depletion of factor H from the plasma converts the sheep erythrocytes into activator surfaces (ctrl, empty circle). Addition of heat inactivated serum to depleted plasma as a source of factor H has a dose-dependent protective effect ("control"). Compared to normal human serum, sera of patient P1 to P3 with CFHR1/CFHR3 deficiency showed less protective activity as indicated by the increased lysis of erythrocytes. The classical pathway of complement activation was inhibited by the chelating agent EGTA. Erythrocytes incubated in buffer do not lyse (ctrl, filled circle). Mean values and standard deviations of three separate experiments are shown.

Ctrl, control

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Jena. A total of 121 aHUS patients from Jena were included in this study. Of these, 19 were found to be deficient in CFHR1 and CFHR3. Genomic deletion of *CFHR1* and *CFHR3* genes was determined in three patients (Table 2). None of these three patients carried a hybrid *CFHR1/CFHR3* gene. The study was approved by the Research Ethics Committee of the University of Cologne, Germany, and by the Research Ethics Board of the Hospital for Sick Children, Toronto, Canada.

Sera and Western blot analysis. Serum samples from 121 aHUS patients (Jena cohort) and 100 anonymous healthy blood donors were assayed for the presence and mobility of factor H and CFHR1. Serum was separated by SDS-PAGE, transferred onto a membrane, and incubated with polyclonal factor H antiserum (Calbiochem-Novabiochem, <http://www.emdbiosciences.com>) or monoclonal C18 antibody [19], which identifies an epitope in factor H and CFHR1. CFHR3 was detected with polyclonal CFHR3 antiserum [20].

Genetic analysis. Genomic DNA was prepared from peripheral blood cells of three patients. Genomic DNA was amplified by PCR using specific primers R1–R8 that cover the 100-kb region downstream of the factor H gene (Table 3). Amplification of the

breakpoint region and sequence analysis was performed using primers P9.2 and B1 and B2. For amplification of segment B and B', primer P was used. The sequence of the amplified products was determined using an ABI 3100 sequence analyzer (Applied Biosystems, www.appliedbiosystems.com). The sequences were compared to that of the genomic DNA of Chromosome 1. The sequences of segment B and segment B' were compared and analyzed for repeat content using CENSOR (<http://www.girinst.org>).

Erythrocyte lysis assay. Factor H-depleted human plasma was prepared by immunoadsorbance of factor H from normal human plasma. Polyclonal factor H antiserum (Merck Biosciences, <http://www.merckbiosciences.co.uk>) was covalently coupled to a 1-ml HiTrap NHS (N-hydroxy succinimide) column (GE Health Care, <http://www.gehealthcare.com>). Factor H depletion was confirmed by Western blotting, and ELISA showed that about 66% of the protein was removed. Depleted plasma was directly used for hemolytic assays. Hemolytic experiments were performed in VBS buffer (veronal buffered saline, 144 mM NaCl, 7 mM MgCl₂, and 10 mM EGTA, pH 7.4). Increasing amounts of heat-inactivated (10 min at 56 °C) CFHR1/CFHR3-deficient serum or control serum were added to the depleted plasma and 2×10^7 sheep erythrocytes. Following incubation at 37 °C for 30 min, the mixture was cleared by centrifugation and the absorbance in the supernatants was measured at 414 nm. Using human complement active plasma sheep erythrocytes serve as non-activators of complement. These cells are protected from lysis and no hemoglobin is released. However, when factor H is depleted from human plasma, this plasma has the ability to lyse sheep erythrocytes, as demonstrated by the release of hemoglobin and an increase in absorbance. Addition of purified factor H or normal human heat-inactivated serum reconstitutes protection of erythrocytes [15].

Multiplex ligation-dependent probe amplification. The MLPA reaction has been previously described [13]. In this study, a completely synthetic probe set was used, obviating the need for a cloning step in the production of probes. Probes were designed to determine dosage for exons 2 and 3 of *CFHR1*, along with control probes for *MSH2* exon 1 and *MLH1* exon 19. Each probe pair hybridises to immediately adjacent targets at the sequence of interest. Hybridisation sequences are shown in Table 4. Probe pairs also contain binding sites for primers used in the MLPA reaction, as well as stuffer sequence to ensure that each amplified probe product is of a unique length. Oligonucleotides were obtained from TAG Newcastle, (<http://www.vhbio.com>). Righthand probes were 5'-phosphorylated and purified by desalting.

Reagents for the MLPA reaction were purchased from MRC-Holland (<http://www.mrc-holland.com>). The ligation reactions were carried out according to the manufacturer's recommended protocol using 100–200 ng of genomic DNA and 2 fmol of probe. Incubations and PCR amplification were carried out on a DNA Engine Tetrad 2 thermal cycler (<http://www.bio-rad.com>). Amplified products were diluted 10-fold to give peak heights within the quantitative range (approximately 100–4,000 units) on the ABI PRISM 3130 Genetic Analyzer capillary electrophoresis system (Applied Biosystems, <http://www.appliedbiosystems.com>). Diluted product (1 µl) and 0.5 µl of ROX 500 internal size standard (Applied Biosystems) were made up to 10 µl using dH₂O and samples were run on the ABI 3130. Peak areas for each sample were determined using the proprietary Genemapper software (Applied Biosystems) and dosage quotients calculated.

Table 2. Profile of Three Patients Deficient in *CFHR1* and *CFHR3*

Patient	Diagnosis	Age [y]	Age at Disease Onset [y]	Factor H [mg/L](230–750)	Therapy	Outcome
P1	D– HUS	14	13 3/12	542	i.v. FFP q 14 days; antihypertensives	No signs of disease activity; renal function slightly decreased; mild hypertension
P2	D– HUS	18	13 10/12	596	Plasmapheresis; i.v. FFP infusion; steroids; antihypertensives	Normal renal function; no chronic treatment required
P3	D– HUS	11	11 1/12	375	i.v. FFP q 14 days; steroids; antihypertensives	No signs of disease activity; renal function improving; mild hypertension

Renal biopsy in all patients showed thrombotic microangiopathy with acute and chronic changes.

D–: no diarrhea at disease onset; FFP: fresh frozen plasma.

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Table 3. Primer Sequences for PCR Amplification and Sequencing

Primer Pairs	Sequence
R1	CATCTGTTGATGGACACTTTGGTGGC GTTGTGAAAAGACAGTCTCTCAATTAAC
R2	GTGACAATTAACATTGTAGAGCAGGGTTG GAATCATATAAACAGAGAGAACTAGAAAGAC
R3	CAATTATTGGTAATGTGTGCACCCTGAAC GTGCAAGATGACGAACCTCGG
R4	GCCCTTTGATTCTTCTTGCCTGATTG GTGGTTATCCAAAGGCAATGCTTACAG
R5	GAGATGGAGTAAGGAAAACAG GGAGAACTAAGAGACAGCGG
R6	GGCATTATACAGTAATAAAGGAGTCAATTCAC GTGTTCACTTTGGAGGTATTTTGTGAGC
R7	CATCTGTTGATGGACACTTTGGTGGC GAGTCTCTGCAATTAACAGTCTTAGG
R8	CGTTTGCCTTGACGTTCCCTGTC GATTGCGCCATTCCACTCCAGTC
P9.2	CCACATGATTGCCTTATACATGC GAGAAAACCTACCAGAGCTAGGCC
B1seq	CCACATGATTGCCTTATACATGC
B2seq	CAAAGACCTTATCTGAATACTTTTACAAC
P	GAAGACGTACCAATGGCCAACAAG GAAGACGTACCAATGGCCAACAAG

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Supporting Information

Accession Numbers

The National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) accession number for the sequences discussed in this paper are *Homo sapiens* Chromosome 1 genomic DNA

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Table 4. MLPA Primers

Primer	Sequence
CFHR1_ex2L	GGGTTCCCTAAGGGTTGGACATAGTTC <i>GTAATAGAAAACCTCCCTGTAGGAACC</i>
CFHR1_ex2R	<i>TGGGAAAATGGCTTATATTTTTCTTCATC</i> TACTCTAGATTGGATCTTCTGGCAC
CFHR1_ex3L	GGGTTCCCTAAGGGTTGGACTAGA
CFHR1_ex3R	<i>TCTGTATCCTGTGTGCAAATAATTTGC</i> ACAGTATCACCTCCAGATGTGTTG <i>ATCTCTAGATTGGATCTTCTGGCAC</i>

MLPA primer binding sites are shown in bold, exon-specific sequences in red, and stuffer sequence in italics.

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(NT_004487.18/Hs1_4644), *MLH1* (NM_000249), and *MSH2* (NM_000251).

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Author contributions. ME, SH, MJ, HR, DR, LS, and AEH performed the experiments. PFZ, JAG, THJG, and CS conceived and designed the experiments and analyzed the data. JM, BH, CL, and THJG took care of the patients, contributed material, and discussed data. PFZ, THJG, and CS wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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