Atypical Haemolytic Uraemic Syndrome Associated with a Hybrid Complement Gene

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Abbreviations: aHUS, atypical haemolytic uraemic syndrome; LCR, low copy repeat; MLPA, multiplex ligation-dependent probe amplification; RCA, regulator of complement activation; SCR, short consensus repeat

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ABSTRACT

Background

Sequence analysis of the regulators of complement activation (RCA) cluster of genes at chromosome position 1q32 shows evidence of several large genomic duplications. These duplications have resulted in a high degree of sequence identity between the gene for factor H (CFH) and the genes for the five factor H-related proteins (CFHL1–5; aliases CFHR1–5). CFH mutations have been described in association with atypical haemolytic uraemic syndrome (aHUS). The majority of the mutations are missense changes that cluster in the C-terminal region and impair the ability of factor H to regulate surface-bound C3b. Some have arisen as a result of gene conversion between CFH and CFHL1. In this study we tested the hypothesis that nonallelic homologous recombination between low-copy repeats in the RCA cluster could result in the formation of a hybrid CFH/CFHL1 gene that predisposes to the development of aHUS.

Methods and Findings

In a family with many cases of aHUS that segregate with the RCA cluster we used cDNA analysis, gene sequencing, and Southern blotting to show that affected individuals carry a heterozygous CFH/CFHL1 hybrid gene in which exons 1–21 are derived from CFH and exons 22/23 from CFHL1. This hybrid encodes a protein product identical to a functionally significant CFH mutant (c.3572C>T, S1191L and c.3590T>C, V1197A) that has been previously described in association with aHUS.

Conclusions

CFH mutation screening is recommended in all aHUS patients prior to renal transplantation because of the high risk of disease recurrence post-transplant in those known to have a CFH mutation. Because of our finding it will be necessary to implement additional screening strategies that will detect a hybrid CFH/CFHL1 gene.

The Editors’ Summary of this article follows the references.
H-related proteins—are similar in that they consist of repetitive units (proteins [4,5]. The secreted protein products of these genes contain tyrosine, valine, and glutamine residues at positions 1,058, 1,191 and 1,197 (encoded by triplets TAT, GAG, and CAA), whilst CFHL1 has histidine, leucine, and glutamic acid residues at positions 157, 159, and 175 (encoded by triplets CAT, GTG and GAA). CFH SCR 19 and CFHL1 SCR 4 consist of 61 amino acids. The exons encoding these two SCRs differ by five bases. Three result in an amino acid difference. CFH has tyrosine, valine, and glutamine residues at positions 1,058, 1,060, and 1,076, respectively (encoded by triplets TAT, GTG, and CAA), whilst CFHL1 has histidine, leucine, and glutamic acid residues at positions 157, 159, and 175 (encoded by triplets CAT, GTG and GAA). CFH SCR 18 and CFHL1 SCR 3 consist of 59 amino acids. At the nucleotide level the exons encoding these two SCRs differ by five bases. Three result in an amino acid difference. CFH has tyrosine, valine, and glutamine residues at positions 1,058, 1,060, and 1,076, respectively (encoded by triplets TAT, GTG, and CAA), whilst CFHL1 has histidine, leucine, and glutamic acid residues at positions 157, 159, and 175 (encoded by triplets CAT, GTG and GAA). CFH SCR 19 and CFHL1 SCR 4 consist of 61 amino acids. The exons encoding these two SCRs differ at one nucleotide position, which does not result in a coding change. CFH SCR 20 and CFHL1 SCR 5 consist of 67 amino acids. The exons differ at two nucleotide positions, both of which affect the amino acid sequence of the encoded proteins; CFH has serine and valine residues at positions 1,191 and 1,197 (encoded by triplets TCG and GTT) while CFHL1 has leucine and alanine residues at positions 290 and 296 (encoded by triplets TGG and GCT). Mutations reported in association with aHUS include c.3572C>T, which results in the change S1191L and c.3590T>C, which results in V1197A, either singly or in combination [6–8]. This raised the possibility that gene conversion of CFH SCR 20 by SCR 5 of CFHL1 is the mutational mechanism involved. LCRs such as those seen in the RCA cluster not only predispose to gene conversion events but are also associated with genomic rearrangements [11]. These rearrangements usually result from nonallelic homologous recombination between LCRs. If nonallelic recombination should occur between the duplicated segments B and B′ shown in Figure 1B, a variety of products are possible (Figure 1C and 1D). If recombination occurred after the terminal exons of CFH and CFHL1 deletion of CFHL1 and CFHL3 would result. If recombination occurred within the region containing the three terminal exons (and their flanking introns) of CFH and CFHL1, then not only would CFHL3 be deleted, but a hybrid CFH/CFHL1 gene would be formed. If, for example, the recombination occurred at “X” in Figure 1B, a hybrid gene would result that consisted of the first 21 exons of CFH (encoding SCRs 1–18 of the hybrid gene) and the last two exons of CFHL1 (encoding SCRs 19 and 20 of the hybrid gene) (Figure 1C). The hybrid gene would encode a protein product identical to the aforementioned S1191L/V1197A mutant, a change that we have shown to be functionally significant [10]. If the recombination occurred at Y, CFHL3 and CFHL1 would be deleted, but CFH would remain intact (Figure 1C). The recombination event would also potentially lead to duplica-

**Hybrid Complement Gene and Atypical HUS**

**Figure 1.** Genomic Structure of the Region of the RCA Cluster Containing the Genes Encoding Factor H and the Five Factor H-Related Proteins

Genomic duplications including the different exons of the six genes were originally determined by Male et al. [4] and are colour-coded. Exons are indicated as vertical lines.

(A) Position of the genes encoding factor H and the factor H-related proteins in a centromeric segment of the RCA cluster at 1q32. (B and C) Nonhomologous recombination occurring at X would result in a hybrid gene consisting of the first 21 exons of CFH (encoding SCRs 1–18 of the hybrid gene) and the last 2 exons of CFHL1 (encoding SCRs 19 and 20 of the hybrid gene). If the recombination occurred at Y this would result in deletion of CFHL3 and CFHL1 but CFH would remain intact.

(D) The recombination event would also potentially lead to a duplication of CFHL1 and CFHL3.

(Figure adapted from Figure 1 of [15] with kind permission of Human Mutation C 2006, Wiley Liss Inc., A Wiley Company.)

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**Introduction**

Atypical HUS (aHUS) is characterised by the triad of a microangiopathic haemolytic anaemia (Coombs’ test negative), thrombocytopenia, and acute renal failure in the absence of a preceding diarrhoeal illness. aHUS can be either sporadic or, if more than one member of a family is affected, familial. In 1998 we established linkage in three families with atypical haemolytic uraemic syndrome (aHUS) to a 26 cM region at chromosome location 1q32 [1]. This area contains a group of genes that play a pivotal role in the regulation of complement activation (the regulators of complement activation [RCA] cluster). In one of these families we found a mutation in the gene encoding the soluble regulator complement factor H [1] and subsequently in another we found a mutation in the gene encoding membrane cofactor protein, a transmembrane regulator [2]. In the third family extensive screening of genes within the RCA cluster failed to reveal an abnormality.

Complement genes within the RCA cluster are arranged in tandem within two groups [3]. In a centromeric 360 kb segment lie the genes encoding factor H (CFH) and five factor H-related proteins—CFHL1–5 (aliases CFHR1–5) (Figure 1A). Sequence analysis of this region shows evidence of several large genomic duplications, also known as low copy repeats (LCRs), resulting in a high degree of sequence identity between CFH and the genes for the five factor H-related proteins [4,5]. The secreted protein products of these genes are similar in that they consist of repetitive units (~60 amino acids) and contain short consensus repeats (SCRs) or complement control protein modules. Each SCR is generally encoded by a single exon. CFH consists of 20 SCRs and CFHL1 consists of 18. The highest degree of sequence identity is seen between SCRs 18 and 20 of CFH and SCRs 3 and 5 of CFHL1. CFH SCR 18 and CFHL1 SCR 3 consist of 59 amino acids. At the nucleotide level the exons encoding these two SCRs differ by five bases. Three result in an amino acid difference. CFH has tyrosine, valine, and glutamine residues at positions 1,058, 1,060, and 1,076, respectively (encoded by triplets TAT, GTG, and CAA), whilst CFHL1 has histidine, leucine, and glutamic acid residues at positions 157, 159, and 175 (encoded by triplets CAT, GTG and GAA). CFH SCR 19 and CFHL1 SCR 4 consist of 61 amino acids. The exons encoding these two SCRs differ at one nucleotide position, which does not result in a coding change. CFH SCR 20 and CFHL1 SCR 5 consist of 67 amino acids. The exons differ at two nucleotide positions, both of which affect the amino acid sequence of the encoded proteins; CFH has serine and valine residues at positions 1,191 and 1,197 (encoded by triplets TCG and GTT) while CFHL1 has leucine and alanine residues at positions 290 and 296 (encoded by triplets TGG and GCT). Mutations reported in association with aHUS include c.3572C>T, which results in the change S1191L and c.3590T>C, which results in V1197A, either singly or in combination [6–8]. This raised the possibility that gene conversion of CFH SCR 20 by SCR 5 of CFHL1 is the mutational mechanism involved. LCRs such as those seen in the RCA cluster not only predispose to gene conversion events but are also associated with genomic rearrangements [11]. These rearrangements usually result from nonallelic homologous recombination between LCRs. If nonallelic recombination should occur between the duplicated segments B and B′ shown in Figure 1B, a variety of products are possible (Figure 1C and 1D). If recombination occurred after the terminal exons of CFH and CFHL1 deletion of CFHL1 and CFHL3 would result. If recombination occurred within the region containing the three terminal exons (and their flanking introns) of CFH and CFHL1, then not only would CFHL3 be deleted, but a hybrid CFH/CFHL1 gene would be formed. If, for example, the recombination occurred at “X” in Figure 1B, a hybrid gene would result that consisted of the first 21 exons of CFH (encoding SCRs 1–18 of the hybrid gene) and the last two exons of CFHL1 (encoding SCRs 19 and 20 of the hybrid gene) (Figure 1C). The hybrid gene would encode a protein product identical to the aforementioned S1191L/V1197A mutant, a change that we have shown to be functionally significant [10]. If the recombination occurred at Y, CFHL3 and CFHL1 would be deleted, but CFH would remain intact (Figure 1C). The recombination event would also potentially lead to duplica-
tion of CFHL1 and CFHL3 (Figure 1D). There is already evidence that nonallelic recombination leading to deletion of CFHL1 occurs as a common polymorphism in the general population [12], and we here show that this phenomenon also leads to the formation of a hybrid CFH/CFHL1 gene associated with aHUS. In particular, we have shown the presence of such a hybrid gene in the affected members of the remaining unsolved family from our original linkage study [1].

Methods

Clinical Details and Informed Consent

The family that is the subject of our study was first reported in 1975 [13] and more recently in 1998 [1]. This is the aforementioned one remaining unsolved family from our original linkage study in three aHUS families in 1998 [1]. The current pedigree is shown in Figure 2. Affected member I:2 died at the age of 57 y, 7 d after presenting with pericarditis, heart failure, and hypertension.

II:1 presented with the classical features of HUS at the age of 45 y, following a 9 mo history of hypertension. Serum complement levels were normal. Renal function did not recover and haemodialysis was commenced. Bilateral nephrectomy was undertaken because of ongoing haemolysis and severe hypertension. He subsequently received two renal transplants, one at the age of 47 y and the other at 60 y. Both kidneys were lost within weeks of transplantation due to recurrent HUS. II:7 and II:10 both died of acute renal failure. II:8 died at the age of 82 y without any evidence of renal disease.

III:3 is currently aged 53 y and is well. Complement profiles have shown a persistently borderline low C3. III:4 presented at the age of 19 y with a grand mal convulsion. She had started an oral contraceptive 6 wk previously. Investigations were consistent with HUS. A complement profile was said to be normal. She died 8 wk after presentation following cardiac arrest. III:5 presented with HUS at the age of 5 y and died 6 d after presentation. III:6 presented at the age of 28 y with a short history of headaches and lethargy. Investigations were compatible with a diagnosis of HUS. He has remained on haemodialysis since then and is currently well. III:8 presented at the age of 7 y with fluid retention, hypertension, and acute renal failure. He died 9 d after admission.

IV:1 presented at the age of 7 y with HUS following a viral illness. Despite treatment with peritoneal dialysis, plasma exchange, and prostacyclin, he did not recover renal function. He received a cadaver renal transplant at age 16 y, which, despite daily plasma exchange, was lost to recurrent HUS within the first two postoperative weeks. He was subsequently treated with both peritoneal dialysis and haemodialysis. He died suddenly at the age of 22 y.

II:2, the twin brother of II:1, presented at the age of 46 y with acute renal failure and hypertension. Investigations were compatible with a diagnosis of HUS. As with his brother, bilateral nephrectomy was necessary to control ongoing haemolysis and hypertension. He subsequently received two transplants, one at the age of 47 y and the other at 60 y. Both kidneys were lost within weeks of transplantation due to recurrent HUS. II:7 and II:10 both died of acute renal failure. II:8 died at the age of 82 y without any evidence of renal disease.

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Figure 3. cDNA Evidence of a Hybrid Gene

(A) Inverted CFH exons 20–23 cDNA sequence showing the site of the first-round forward primers (blue), the second round forward primers (green), and the reverse primers for both rounds (red). The nucleotides at which the CFH and CFHL1 sequences differ are shown in bold and highlighted (excluding exon 20).

(B) An electropherogram showing the three primer sites.

(C) Sequence analysis showing the genetic variations c.3590T>C and c.3572C>T.

II:3
II:6
II:1
II:7
Control
Mutation screening of the genes for factor H, membrane cofactor protein, and factor I in this family had not revealed any abnormalities.

The study was approved by the Northern and Yorkshire Multi-Centre Research Ethics Committee and informed consent obtained.

**Complement Assays**

Convalescent EDTA plasma samples were obtained and stored at −80 °C. C3 and C4 levels were measured by rate nephelometry (Beckman Array 360; Beckman, Fullerton, California, United States). Factor H levels were measured by radioimmunodiffusion (Binding Site, Birmingham, UK).

### CFH Genomic DNA Sequencing

CFH gDNA sequencing was undertaken as previously described [6] apart from exon 23, where the primers used were: forward 5′-CTGTAGCGCGACGGCCAGT-3′ and reverse 5′-CAACCGTTAGTTTTCCAGGA-3′. All primers incorporated a 5′ “UNISEQ” M13-derived 17 bp tail that allowed subsequent sequencing using a common forward (5′-GTTACCGCAGGGCCGT-3′) and reverse (5′-CAGGCCG-CAGCGATGAC-3′) primer. PCR products were purified using magnetic microparticles (AMPure, Beckman) to remove unincorporated dNTPs, primers, and salts. Sequencing reactions were carried out by dye terminator cycle sequencing (DTCS kit, Beckman) using UNISEQ primers, purified using magnetic microparticles (CleanSeq, Beckman), and electrophoresed on a fluorescent 16 capillary sequencer (Beckman CEQ 8000).

The reference nucleotide sequence for CFH was taken from GenBank RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) file NM_000186.1, and the nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1. The factor H amino acid numbering includes the 18-residue signal peptide. The reference nucleotide sequence for CFHL1 was taken from Genbank RefSeq file NM_002113.1, and the nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1. CFH exons have been numbered 1–23 according to Rodriguez de Cordoba et al. [14]. In this nomenclature, exon 10 contributes to the transcript for factor H-like protein 1 but not factor H. The factor H-related protein 1 amino acid numbering includes the 18-residue signal peptide.

### CFH cDNA Sequencing

For cDNA sequencing of CFH (Figure 3) mRNA was extracted from peripheral blood lymphocytes of family members (affected and unaffected) and unrelated controls. cDNA was prepared in a standard manner and CFH exons 21–23 amplified by seminested PCR. The forward primers were designed to be specific for CFH and were therefore sited in exon 20, which is not homologous to CFHL1. The reverse primer was complementary to both exon 23 of CFH and exon 6 of CFHL1 (Figure 3A). The first-round forward primer was 5′-GCTATACCCATGAGAGAGAC-3′ and the second-round forward primer was 5′-GGGATGAGCATGGCTA-3′. The reverse primer for both rounds was 5′-GGGATGAGCAGAATGCCTGACT-3′. Each primer incorporated a “UNISEQ” tail as before to enable sequencing with UNISEQ primers.

### Identification and Screening of the Breakpoint in CFH/CFHL1 Hybrid Gene

To identify and screen the breakpoint (Figure 4), first we amplified genomic DNA across the intron between exons 21 and 22 (CFHL1 exon 5) of the CFH/CFHL1 hybrid gene using primers specific for CFH (forward) and CFHL1 (reverse) sequence.

Next, we screened the identified breakpoint in the affected family using the primer pair 5′-CCATCTGGTGTAGAGGACGTTGACTGT-3′ (forward) and 5′-ACCCAGAATCAGTGCTGC-3′ (reverse) (Figure 4B), which anneal to both CFH and CFHL1. Each primer incorporated a “UNISEQ” tail as before to enable sequencing with UNISEQ primers.

### CFH Dosage Analysis by Quantitative Fluorescent PCR

Two multiplex PCR assays were designed to simultaneously amplify exons 20–23 of CFH. PCRs were carried out in 25 μl volumes using 150 ng of DNA and contained 0.5 mM each dNTP, 6.7 mM MgCl2, 12.5 pmol of each primer, 1 U of a hot start Taq polymerase (Immolase, Bioline) in a buffer of 16 mM (NH4)2SO4, 67 mM Tris-HCl, 0.01% Tween-20. PCR cycling conditions were such that amplification remained in the linear phase of the reaction (95°C for 7 min; 20 cycles of:

**Table 1. Primer Sequences for the CFH QF-PCR Dosage Analysis**

| Exon      | Forward                               | Reverse                              | Basepairs |
|-----------|---------------------------------------|--------------------------------------|-----------|
| CFH exon 20 | GAAACAGATTGTCTCAGTTTACCCT            | GCTGGTCCTCGAAGCTCTGGACGCT            | 308       |
| CFH exon 21 | CTAGTTGAGAACCTCTTTTTTTCTAT           | GGAAGATTGATATTTTTACACCCACCATCAG      | 354       |
| CFH exon 22 | TTTCCTCAGGACTCACTTCTTCTCTCT          | CTGTTGATTTTGTACAAACAGTG             | 407       |
| CFH exon 23 | GATGGTCTACATAGTGTTGGAT              | AGTCTCGAATAAGGTTGTCAC               | 379       |
| MLH1 exon 14 | TGTCCTACTGTTTGTGTCTCTGGTG            | CTACCTGATCGCTGCCTCCTTACG            | 298       |
| BRCA1 exon 16 | AATTTTACAGAGACACAGAC                | AAAACATTTTCCAGAGATGTTG              | 450       |

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Figure 4. Identifying and Screening the Breakpoint Region

(A) Sequence of unique PCR product generated with specific CFH (forward) and CFHL1 (reverse) primers from III:6 (affected) demonstrating the hybrid product. Unique CFH positions are indicated with black arrows, and unique CFHL1 nucleotides are indicated with red arrows.

(B) The genomic sequence of CFH is shown aligned above CFHL1. Exons 21 and 4 of the two genes respectively are highlighted in grey. The primer-binding sites for the PCR are shown in red. The differences visible in intron 21/4 from sequencing the product are highlighted in the standard base colours A (green), C (blue), G (black), and T (red). The breakpoint is within the region underlined.

(C) Sequence of the intron between exons 21 and 22 shows a switch from heterozygosity at CFH/CFHL1 unique bases to a CFHL1 sequence in III:3 (unaffected carrier) and III:6 (affected) compared with III:1 (unaffected), III:7 (unaffected), and a normal unrelated control.

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94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min; and a final extension of 10 min at 72 °C). Primers that amplify MLH1 exon 14 and BRCA1 exon 16 were included in the assay as controls for normal dosage. One of each pair of primers was fluorescently labelled (5’FAM), and all primers are shown in Table 1. After PCR amplification products were analysed by capillary electrophoresis (ABI, Perkin Elmer). Peak areas were obtained for each sample and dosage quotients calculated.

**CFH Multiplex Ligation-Dependent Probe Amplification**

The multiplex ligation-dependent probe amplification (MLPA) reaction has been described [15]. 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 a range of CFH exons, along with control probes for MSH2 exon 1 and MLH1 exon 19 (NM_000251, NM_000249). Each probe pair hybridises to immediately adjacent targets at the sequence of interest. Hybridisation sequences are shown in Figure 5 and Table 2. Probe pairs also contained 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 Limited (Gateshead, UK [now available at VH Bio: http://www.vhbio.com/home.aspx]). 5’ probes were RP-column purified. 3’ probes were 5’-phosphorylated and purified by desalting.

Reagents for the MLPA reaction were purchased from MRC-Holland (Amsterdam, the Netherlands). 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 amplifications were carried out on a DNA Engine Tetrady 2 thermal cycler (BioRad Laboratories, Hercules, California, United States). Amplified products were diluted 1 in 10 to give peak heights within the quantitative range (approximately 100–4,000 units) for size standards, 1 µl of ROX 500 internal size standard 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 and dosage quotients calculated.

**Southern Blots**

To provide further confirmation that the point of recombination responsible for the CFH/CFHL1 hybrid gene lies within the intron between CFH exons 21/22 and CFHL1 exons 4/5, Southern blots were done using a 1.1 kb probe (Figure 6). The putative crossover leading to the formation of the CFH/CFHL1 hybrid gene is shown as a line that crosses over 110–150 bases into the introns between exons 21/22 of CFH and 4/5 of CFHL1. A DNA probe was prepared by PCR amplification of a 1.1 kb region spanning the putative breakpoint using the following primers: forward, 5’-GTACCGCGGCGCCAGTGGTCTACAGGTTCTACCAG-GAGGA-3’ and reverse, 5’-GTGCACCTATAACAGGCGCAT-3’.

Genomic DNA (10 µg) from two affected individuals, one unaffected individual, and two individuals who were homozygously deleted for CFH1 was digested with HindIII. Electrophoresis was carried out overnight in a 0.8% agarose gel in 1× TAE buffer (0.04 M Tris-acetate buffer, 0.001 M EDTA) at 40 mA. DNA was transferred to a nylon membrane (Hybond N+, Amersham [http://www.amersham.com/]) after denaturation in 1.5 M NaCl, 0.5 M NaOH and neutralisation in 0.4 M Tris base, 0.25 M trisodium citrate, 2.5 M NaCl. The DNA probe (20 ng) was labelled using the Rediprime system (Amersham). Hybridisations were carried out overnight at 65 °C in a mix containing 5× SSC (0.75 M NaCl, 0.075 M trisodium citrate), 5× Denhardt’s (0.1% PVP, 0.1% BSA, and 0.1% Ficoll), 0.1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulfate, and 100 µg/ml sonicated denatured salmon sperm DNA. The filter was washed at 65 °C to a final stringency of 0.5× SSC (including 0.1% SDS) and autoradiographed with intensifying screens for 12 h.

**Results**

**Complement Profiles**

Serum levels of C3, C4, and factor H are shown in Table 3. Factor H levels were normal in all family members, as was expected, because the secreted product of the hybrid gene is identical to the factor H mutant c.3572C>T, S1191L/c.3590T>C, V1197A.

**CFH gDNA Sequencing**

Sequencing of genomic CFH DNA from II:8 (unaffected carrier), III:1 (unaffected), III:3 (unaffected carrier), III:6 (affected), III:7 (unaffected) and IV:1 (affected) showed wild-type sequence. This was expected, because the hybrid CFH/CFHL1 gene described here would not be picked up by genomic sequencing using CFH-specific primers.

**CFH cDNA Sequencing**

In III:3 (unaffected carrier) and III:6 (affected), cDNA sequencing showed two heterozygous changes c.3572C>T and c.3590T>C in exon 23, leading to S1191L and V1197A, respectively (Figure 3B and 3C). In addition, there was a heterozygous synonymous change in exon 22. These changes were not found in III:1, III:7 (both unaffected), or an unrelated control.

**Identification and Screening of the Breakpoint in the CFH/CFHL1 Hybrid Gene**

The DNA breakpoint in III:6 (affected) was identified by sequencing PCR products using specific CFH (forward) and CFHL1 (reverse) primers spanning the intron between exons 21 and 22 (CFH 5’ exon 5) of the CFH/CFHL1 hybrid gene. This generated a unique product, sequencing of which
showed the breakpoint to be in a 52 bp section starting 118 bp into intron 21. The 52 bp section is defined by differences between \textit{CFH} and \textit{CFHL1} sequence (Figure 4A). Screening of other family members using primers spanning the breakpoint and designed to anneal to both \textit{CFH} and \textit{CFHL1} (Figure 4B) confirmed the breakpoint. Sequence of this region (Figure 4C) shows a switch from heterozygosity at \textit{CFH}/\textit{CFHL1} unique bases to \textit{CFHL1} sequence in III:3 (unaffected carrier) and III:6 (affected) compared with III:1 (unaffected), III:7 (unaffected), and a normal unrelated control.

\textit{CFH} Dosage Analysis by Quantitative Fluorescent PCR and MLPA

\textit{CFH} dosage quotients showed an apparent heterozygous “deletion” of \textit{CFH} exons 22 and 23 in II:8 (unaffected carrier), III:3 (unaffected carrier), III:6 (affected), and IV:1 (affected). There was no evidence of a “deletion” in III:1 and III:7 (both unaffected) (Figure 7).

In a panel of 80 patients with aHUS, three further individuals were identified by MLPA as having a deletion of \textit{CFH} exons 22 and 23. In all three, factor H levels were normal and \textit{CFH} gDNA sequencing had shown normal wild-type sequence, but presence of a hybrid \textit{CFH}/\textit{CFHL1} gene was confirmed by \textit{CFH} cDNA sequencing. This showed the same changes identified in III:3 and III:6.

In a panel of 100 normal control individuals, MLPA showed no evidence of a deletion of \textit{CFH} exons 22 and 23.

Southern Blot

In III:3 (unaffected carrier) and III:6 (affected) a Southern blot with HindIII showing an additional 8.6 kb band (the band at “B” in Figure 6B). This additional band is derived from the hybrid \textit{CFH}/\textit{CFHL1} gene as shown in Figure 6A.

Discussion

In this study we have provided conclusive evidence that LCR nonhomologous recombination in the RCA cluster of genes has resulted in the formation of a hybrid \textit{CFH}/\textit{CFHL1} gene that predisposes to the development of aHUS. The protein product of the hybrid gene is identical to the factor H mutant c.3572C>T, S1191L/c.3590T>C, V1197A which we have shown arises by gene conversion in aHUS [10]. We have previously shown that this mutant is functionally significant, in that binding to C3b is impaired. The hypothesis for the existence of a hybrid \textit{CFH}/\textit{CFHL1} gene in the family described in this manuscript was based on two observations. First, we knew from our original linkage study [1] that the affected individuals in this family mapped to the RCA cluster at position 1q32. Moreover, in this family there was a strong history of disease recurrence following kidney transplantation, suggesting that a soluble circulating complement regulator was responsible. Second, our recent observation that LCRs in the RCA cluster predisposed to gene conversion events suggested that LCR nonhomologous recombination
Figure 6. Southern Blot Evidence of Genomic Rearrangement

(A) A Southern blot using a 1.1 kb probe overlying CFH exon 21 and CFHL1 exon 4 hybridised to HindIII-digested DNA (sites shown as arrows) will result in fragments of 11.2 kb from CFH, 1.4 kb from CFHL1, and 8.6 kb from a CFH/CFHL1 hybrid gene. The site of the 1.1 kb probe is indicated above.

(B) Southern blot showing an additional 8.6 kb band (indicated by B) in lanes 2 and 3, which represent III:3 (unaffected carrier) and III:6 (affected) compared to lanes 1, 4, and 5, which represent III:1 (unaffected) and individuals with homozygous deletion of CFHL1. Bands at A and C represent fragments of 11.2 kb from CFH and 1.4 kb from CFHL1, respectively. A size ladder is shown to the right with heavy arrows indicating the expected sizes.

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Table 3. Complement Levels

| Family Member Identifier | C3 (0.68–1.80 g/l) | C4 (0.18–0.60 g/l) | Factor H (0.35–0.59 g/l) |
|--------------------------|--------------------|--------------------|-------------------------|
| III:1                    | 1.60               | 0.29               | 0.75                    |
| III:3                    | 0.69               | 0.19               | 0.53                    |
| III:6                    | 0.55               | 0.22               | 0.53                    |
| III:7                    | 1.41               | 0.30               | 0.58                    |
| IV:1                     | 1.06               | 0.20               | 0.68                    |

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Therefore, now recommend that all patients be screened also for CFH genomic sequencing using CFH/CFHL1 genotyping prior to transplantation. However, the hybrid gene might also be occurring. In support of this was the observation that the presence of factor H-related protein 1 (encoded by CFHL1) in the serum of normal controls is polymorphic, with 4.4% of healthy blood donors having complete deficiency [12]. We hypothesised that the crossover responsible for deletion of CFHL1 could also potentially result in formation of a CFH/CFHL1 hybrid gene. The evidence presented here strongly supports this hypothesis. Moreover, we have evidence from three other unrelated aHUS patients that the phenomenon is not unique to this family. Renal transplantation in patients with a CFH mutation is associated with an 80% risk of the graft being lost to recurrent disease within two years of transplantation [16]. It is currently recommended that all patients undergo CFH genotyping prior to transplantation. However, the hybrid CFH/CFHL1 gene described here would not be picked up by genomic sequencing using CFH-specific primers. We would, therefore, now recommend that all patients be screened also with CFH MLPA.

Could the results of this study have implications for other complement-related diseases? It has been established in several independent cohorts that CFH alleles act as a susceptibility factor for age-related macular degeneration [17–20] and type II membranoproliferative glomerulonephritis [21]. It would be fascinating to test the hypothesis that copy number of CFHL1 and CFHL3 acts as a susceptibility factor for such diseases. It is tempting to speculate that such an effect might be mediated by an interaction between factor H and the factor H-related proteins.

Supporting Information

Accession Numbers

Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) accession numbers for the genes and conditions discussed in this article are aHUS (235400), CFH (134370), CFHL1 (134371), CFHL2 (600889), CFHL3 (605336), CFH-L (OMIM 605337), and CFH-L (OMIM 605337). The GenBank (http://www.ncbi.nlm.nih.gov) accession numbers for other genes are MSH2 exon 1 (NM_000251) and MLH1 exon 19 (NM_000249).

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Editors’ Summary

Background. Atypical hemolytic uremic (aHUS) syndrome is a rare, chronic disease that can run in families. People with the condition are prone to developing kidney failure and high blood pressure, and are likely to have a shorter life span than healthy people. Previous work done by a group of researchers in Newcastle-on-Tyne, UK looked at the genetic underpinnings of aHUS in three families suffering from the condition. They found a region of the genome that was linked with the disease in all three families. That region was known to contain a gene for a protein called “factor H,” as well as a number of other genes for proteins that are involved in the same pathway as factor H in controlling an ancient defence system called complement. This system helps antibodies to kill invaders by marking any cell that is not protected by proteins such as factor H. Our own cells would be under constant threat without protective proteins such as factor H. Later studies found simple genetic mutations in people with aHUS, in the genes coding for factor H. However, other work suggested that in some families with aHUS, simple genetic mutations might not be the cause; instead more complicated rearrangements of the genome might occur which would then result in an abnormal factor H that incorporated part of the gene for another protective protein called factor H related protein 1.

Why Was This Study Done? The researchers knew that it was important to understand the exact genetic mutations linked with aHUS in different families. This was because the exact type of mutation would help them predict whether a kidney transplant is likely to be successful in treating an individual with aHUS who has developed kidney failure. In people with mutations affecting proteins produced by the kidney, a kidney transplant would be likely to work; but in people with mutations affecting factor H, which is produced by the liver, the disease would probably recur after a kidney transplant.

What Did the Researchers Do and Find? In this study, the researchers went back to one of the three families with aHUS they had previously studied. The researchers had shown before that in this family, the disease was linked with the genome region containing factor H, but no precise mutation in that region had been found. This time, the researchers screened the genome of the family members and looked in particular for a specific rearrangement of the genome that they suspected might be involved. They found that the genomes in this family had been shuffled in the factor H region, resulting in an abnormal version of factor H being produced.

What Do These Findings Mean? The mutation these researchers identified is likely to result in development of aHUS that does not get better after a kidney transplant, because the abnormal factor H would still be produced in the liver after a transplant had been done. Therefore, the researchers suggest that patients with aHUS be checked for this particular mutation before it is decided whether to go ahead with a transplant.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0030431.
- US National Institutes of Health Office of Rare Diseases information about atypical hemolytic uremic syndrome
- The Online Mendelian Inheritance in Man (OMIM) contains an entry on hemolytic uremic syndrome. OMIM is a database of human genes and genetic disorders developed by the US National Center for Biotechnology Information
- The US National Kidney and Urologic Diseases has a page about hemolytic uremic syndrome
- The Wikipedia has a page about HUS (note that Wikipedia is a free online encyclopedia that anyone can edit)