Factor H Competitor Generated by Gene Conversion Events Associates with Atypical Hemolytic Uremic Syndrome

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ABSTRACT

Atypical hemolytic uremic syndrome (aHUS), a rare form of thrombotic microangiopathy caused by complement pathogenic variants, mainly affects the kidney microvasculature. A retrospective genetic analysis in our aHUS cohort (n=513) using multiple ligation probe amplification uncovered nine unrelated patients carrying a genetic abnormality in the complement factor H related 1 gene (CFHR1) that originates by recurrent gene conversion events between the CFH and CFHR1 genes. The novel CFHR1 mutants encode an FHR-1 protein with two amino acid substitutions, L290S and A296V, converting the FHR-1 C terminus into that of factor H (FH). Next-generation massive-parallel DNA sequencing (NGS) analysis did not detect these genetic abnormalities. In addition to the CFHR1 mutant, six patients carried the previously uncharacterized CFH-411T variant. In functional analyses, the mutant FHR-1 protein strongly competed the binding of FH to cell surfaces, impairing complement regulation, whereas the CFH-411T polymorphism lacked functional consequences. Carriers of the CFHR1 mutation presented with severe aHUS during adulthood; 57% of affected women in this cohort presented during the postpartum period. Analyses in patients and unaffected carriers showed that FH plasma levels determined by the nonmutated chromosome modulate disease penetrance. Crucially, in the activated endothelial (HMEC-1) cell assay, reduced FH plasma levels produced by the nonmutated chromosome correlated inversely with impairment of complement regulation, measured as C5b-9 deposition. Our data advance understanding of the genetic complexities underlying aHUS, illustrate the importance of performing functional analysis, and support the use of complementary assays to disclose genetic abnormalities not revealed by current NGS analysis.


Atypical hemolytic uremic syndrome (aHUS) is an ultra-rare disease characterized by AKI, thrombocytopenia, and microangiopathic hemolytic anemia, which results from an impaired protection of host endothelial cells from complement damage.1 Criteria have been established to facilitate the diagnosis of aHUS.2 Fifty to 70% of patients with aHUS have an underlying inherited and/or acquired complement abnormality.3–11 Missense mutations and complex genomic rearrangements implicating CFH are prototypical of aHUS and the most prevalent genetic alterations, present in >25% of the patients with aHUS, in all series.3 Most aHUS-associated CFH mutations disrupt the C terminus of the protein, a region that is...
critical to the capacity of FH to bind cell surfaces and control local activation of complement. Carriers of these CFH mutations express FH molecules that possess normal regulatory activity in plasma but a limited capacity to bind and protect cells from complement lysis. This understanding of aHUS pathogenesis fits well with the functional consequences of aHUS-associated mutations in other genes like MCP, CFI, C3, or CFB and those of auto antibodies anti-FH; these abnormalities also lead to decreased protection of host cells from complement lysis without significantly affecting complement homeostasis in plasma.

Despite significant advances in our understanding of the pathogenic mechanisms underlying aHUS and the constant technical improvements in genetic testing, we fail to identify a genetic (or acquired) factor in approximately one third of the patients with a clinical diagnosis of aHUS that has excluded other thrombotic microangiopathies. This failure is often justified by arguing the existence of pathogenic variants in genes not included in the genetic analysis and/or the presence of genetic abnormalities that go undetected by current genetic testing strategies.

Here, we report the identification of mutations in the CFHR1 gene that generate by gene conversion events and that are undetectable by the current Next-generation massive-parallel DNA sequencing (NGS) analysis approaches. We demonstrate that the protein encoded by the mutated CFHR1 gene competes FH, disturbing complement regulation on cell surfaces, and that penetrance of aHUS in mutation carriers is modulated, among other factors, by the levels of FH determined by the CFH gene in the nonmutated chromosome.

RESULTS

Identification of a Novel Genetic Abnormality Associated with aHUS

A retrospective multiplex ligation-dependent probe amplification (MLPA) analysis in our aHUS cohort (n=513) resulted in the identification of nine patients sharing a similar pattern consisting in a gain of CFH exon 23 probes and a loss of CFHR1 exon 6 probes. NGS analyses, using a gene panel including all aHUS candidate genes, failed to reveal genetic abnormalities in CFH exon 23 and CFHR1 exon 6 in these patients.

Six of the patients (HUS362, HUS209, HUS323, HUS527, HUS2057, HUS715) share an MLPA pattern in which only one probe shows gain in CFH exon 23 and another shows loss in CFHR1 exon 6 (Figure 1). Sanger sequencing of CFHR1 exon 6 in these patients revealed two genetic variants in heterozygosis causing the amino acid substitutions, L290S and A296V (Figure 2). To further characterize the FHR-1 protein in these individuals, we developed a proteomic strategy and showed that the amino acid substitutions, L290S and A296V, were present in the FHR-1 from these patients (Figure 3, D and E).

Three Different Events of Gene Conversion Generated the Mutant CFHR1 Genes in Our Patients with aHUS

As illustrated above, despite the differences in the MLPA patterns, all nine patients are heterozygous for the amino acid substitutions, L290S and A296V, in CFHR1. These substitutions are relevant to aHUS because they convert the C-terminal region of FHR-1 into that of FH and in this respect the mutant CFHR1 gene identified in our patients is equivalent to the CFHR1::CFH hybrid gene previously described. However, in contrast with the reported CFHR1::CFH hybrid genes that originated by nonhomologous recombination with duplication of the CFHR3 and CFHR1 genes, the mutant CFHR1 genes described here generated by gene conversion events between the CFH and CFHR1 genes without altering the CFHR3 and CFHR1 copy number.

On the basis of the MLPA and DNA sequencing differences observed in our patients, we concluded that the CFHR1 mutant gene in our cohort was generated by three different gene conversion events in which a distinct fragment of the 5’ end of the CFH gene was introduced in the CFHR1 gene (Supplemental Figure 1). The six patients who also carry the CFH S411T variant share one of these gene conversion events. Segregation analysis in families indicates that the CFH S411T variant and the mutant CFHR1 gene segregate together within the same CFH-CFHR1 extended haplotype. Notably, five of these patients originate from the north of Spain and the sixth one is from a nearby region in the north of Portugal, suggesting that all of these patients have a common ancestor.

Significance Statement

Atypical hemolytic uremic syndrome (aHUS) is a rare form of thrombotic microangiopathy associated with complement genetic abnormalities. A retrospective genetic analysis in our aHUS cohort (n=513) identified nine unrelated patients carrying a novel genetic abnormality that causes the transfer of DNA fragments between two complement genes. This genetic abnormality, which escapes detection by currently-used next-generation massive parallel DNA (NGS) sequencing, generates a strong competitor of factor H, the main complement regulator that prevents complement-mediated damage to host tissues. The result is complement dysregulation and endothelial damage. The data advance understanding of the genetic complexities underlying aHUS and illustrate the importance of functional assays to disclose genetic abnormalities not revealed by current NGS analysis.
Functional Analysis of the CFH and CFHR1 Genetic Variants

To evaluate the pathogenicity of the FH-S411T gene variant, the mutant FH protein was purified to homogeneity and its capacity to regulate the complement alternative pathway (AP) was evaluated using a variety of analyses (Supplemental Materials). None of these analyses showed differences between the activities of the mutant FH-411T and the control FH-411S, which clearly establishes that S411T is a CFH polymorphism without functional consequences (Figure 4).

Because the mutant FHR-1 protein presents a C-terminal region that is identical to that of FH, we hypothesized that the FHR-1 mutant could compete binding of FH to its ligands and, therefore, impair the capacity of FH to regulate complement activation on cellular surfaces. To test this possibility, we purified the mutant FHR-1 protein to homogeneity and analyzed its capacity to compete FH in a sheep erythrocyte hemolytic assay. The results demonstrate that the mutant FHR-1 competes in a dose-dependent way the regulation of the complement AP by FH (Figure 5).

Incomplete Penetrance of aHUS in Carriers of the CFHR1 Mutant

Table 1 summarizes the demographic and clinical data of the nine patients carrying the mutant CFHR1 to illustrate that they have severe presentations of the disease with multiple recurrences that, before the eculizumab era, evolved to ESRD. Notably, however, among relatives of these patients we identified 12 asymptomatic carriers of the CFHR1 mutant (Figures 1–3). This incomplete penetrance (43%; 9 of 21) strongly suggests that additional genetic and environmental factors are required for aHUS development. The analysis of the CFH and MCP risk haplotypes in the 21 carriers did not reveal a statistically significant
There is, however, a trend suggesting the CFH-H3 and CFH-H4b haplotypes, associated with lower and higher FH levels, may be overrepresented in patients and unaffected carriers, respectively.

Analyses of FH plasma levels in patients (mean 12.5 mg/dl) and asymptomatic (16.0 mg/dl, respectively; P=0.01) carriers were significantly different, suggesting that the penetrance of the disease is modulated by the FH/mutant FHR-1 ratio. Because most patients and asymptomatic carriers share identical CFH-CFHR1 mutant haplotype, we evaluated next the contribution of the nonmutated CFH-CFHR1 allele to the FH plasma levels. We have previously developed an assay to measure the allele-specific FH expression in individuals that are Tyr402His heterozygotes. Here, we have applied this analysis to 16 of the CFHR1 mutant carriers who were Tyr402His heterozygotes, seven affected and nine asymptomatic relatives. Interestingly, there was a statistically significant difference in the FH plasma levels determined by the CFH allele carried by the nonmutated chromosome between patients (mean 6.5 mg/dl) and asymptomatic (mean 10.4 mg/dl, respectively; P<0.01) carriers of the CFHR1 mutant (Figure 6A).

In addition to genetic factors, the contribution of environmental factors triggering the development of aHUS is also well documented. Consistent with this, the clinical records of the nine patients reveal that disease development is associated with known triggers of aHUS (Table 1). Notably, four of our seven female patients (57%) developed aHUS during pregnancy or the postpartum period.

C5b-9 Deposition on Activated HMEC-1 Cells
We determined next whether the concurrence of the CFHR1 mutant and low FH levels determined by the nonmutated chromosome influence complement dysregulation at endothelial cell level. To this end, human microvascular endothelial cells (HMEC-1) preactivated with ADP were exposed to serum from patients and asymptomatic carriers of the CFHR1 mutant and the surface area covered by C5b-9 deposits was evaluated using confocal microscopy. Serum samples from eight carriers of the CFHR1 mutant, Tyr402His heterozygotes, were available for this analysis. Importantly, the data show an inverse correlation between the levels of FH determined by the nonmutated CFH-CFHR1 haplotype and the C5b-9 deposition on endothelial cells (Figure 6B). As expected, C5b-9

Figure 2. Genetic analysis in the patient with aHUS included in group 2. (A) Pedigree of the patient included in this group. Solid symbol identifies the proband. Small black dots identify individuals presenting the MLPA pattern shown in (B) and the DNA sequences of CFH exon 9 and CFHR1 exon 6 depicted in (C). The CFH haplotypes of each individual are shown, indicating with an asterisk the CFH haplotype that segregates with the abnormal MLPA pattern and the CFHR1 mutant. Haplotype H4b carries the delCFHR3-CFHR1. Numerals indicate the contribution of each CFH haplotype to the total plasma FH levels (milligrams per deciliter). Small gray squares indicate whether the individual is heterozygous (one square) or homozygous (two squares) for the MCPggaac risk haplotype. (B) Illustrative example of the results of the MLPA analysis showing the number of copies for each of the probes within the CFH-CFHRs genomic region. Normal copy numbers are shown with black circles, gains or losses with red circles. (C) Selected fragments of the electropherograms from the DNA Sanger sequencing analysis to show the critical DNA sequences in CFH exon 9 and CFHR1 exon 6 that share all individuals identified by the small black dots. Notice than the T and C nucleotides that identify the normal CFHR1 sequence L290 and A296 are in this case over-represented compared with the electropherograms shown in Figure 1. This is most likely because the primers used in the amplification of CFHR1 exon 6 fail to efficiently amplify the allele carrying the S290 and V296 genetic variants.
deposition using sera from affected individuals who were under eculizumab treatment was normal (Figure 6B).

DISCUSSION

Segmental duplications in the CFH-CFHRs genomic region cannot be properly analyzed by current NGS approaches. For this reason, we set up a retrospective analysis of our aHUS cohort using MLPA and performed Sanger sequencing of these critical regions in all patients. Using this approach, we identified nine patients with aHUS carrying the L290S and A296V amino acid substitutions in heterozygosis in exon 6 of CFHR1. This genetic abnormality has gone undetected in early genetic screening using Sanger sequencing because the CFHR1 gene was not included in the analysis. In six of the nine patients, these early analyses identified the presence of the CFH-411T variant in heterozygosis, although the functional consequences and relevance to aHUS of this amino acid substitution were unknown.

Segregation analyses in the pedigrees of the patients demonstrate that the L290S and A296V amino acid substitutions in
CFHR1 always segregate together, with the same haplotype, and do not associate with genomic rearrangement in this genetic region. This supports that a transfer of sequences from the CFH gene to the CFHR1 gene (gene conversion) is the likely mutational mechanism involved in the generation of this CFHR1 variant. Moreover, our MLPA and Sanger sequencing data support that three different gene conversion events were involved in the generation of the mutated CFHR1 genes found in our nine patients.

We have previously documented several examples of gene conversion events between exon 23 of CFH and exon 6 of CFHR1 in which the CFHR1 sequences have been introduced in the CFH gene. Similarly, there is also robust evidence of major rearrangements in the CFH-CFHR1–5 gene region that result in the deletion or duplication of the CFHR1 and CFHR3 genes and the generation of CFH::CFHR1, CFH::CFHR3, and CFHR1::CFH hybrid genes. However, this is the first time that gene conversion events introducing sequences of CFH into CFHR1 are described. Finding nine patients carrying this genetic abnormality and documenting three different gene conversion events suggest this may not be such a rare genetic alteration among patients with aHUS.

FH is the main regulator of the AP, both in fluid phase and on cellular surfaces. FH is a relatively abundant plasma protein that is essential in restricting the action of complement to the pathogens’ surfaces. The complement regulatory activities of FH are modulated by the FHRs. These proteins lack the complement regulatory domains of FH, but have conserved the FH surface recognition domains, which confers to them the capacity to compete the binding of FH to its ligands in some complement-activating surfaces. This FH antagonistic function of the FHRs is termed complement deregulation.

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**Figure 4.** Functional analysis of the FH-411T variant show it is a polymorphism without functional consequences. (A) Coomassie-stained SDS-PAGE gel (inset) to show that the FH-411S and FH-411T genetic variant were purified to homogeneity. FH concentrations were adjusted on the basis of the absorbance at 280 nm and were confirmed to be identical in the FH-411S and FH-411T preparations by a sandwich ELISA using polyclonal rabbit anti-FH and mouse monoclonal anti-FH antibodies. (B) Hemolytic assays using sheep erythrocytes. We considered that 100% lysis is the percentage of sheep erythrocytes that are lysed (which is approximately 40%) when they are exposed to 20% of a human serum that has been depleted of 75% of the FH. By adding increasing amounts of the FH-411S and FH-411T to the FH-depleted serum we show that both genetic variants have identical capacity to prevent the lysis of the sheep erythrocytes. Data are mean±SD of triplicates. (C) The F1-cofactor activity of the FH-411S and FH-411T genetic variants was assayed in vitro using purified proteins. The graph is a time course experiment showing the % of C3b cleaved, as determined by the α-chain/β-chain ratio, after mixing 200 ng of each FH variant with 56 ng of F1 and 750 ng of C3b. Both FH genetics variants resulted in identical cleavage of the C3b, supporting that their F1-cofactor activities are also identical. Data are mean±SD of triplicates. (D) FH binding to C3b. We inject 1.5 µg/ml of both the FH-411S and FH-411T protein variants to a SPR chip coated with OX24, a mouse mAb that recognizes the N-terminal region of FH, and flow subsequently C3b at a concentration of 36 µg/ml. As shown in the sensogram, both FH genetic variants show identical capacity to bind fluid phase C3b through their C-terminal region. (E) We tested the DAA of the FH-411S and FH-411T genetic variants using SPR. The AP C3-convertase was formed using a C3b-coated CM5 chip by flowing FB and FD as described previously. After a brief period allowing spontaneous decay we flow buffer (dashed line), FH-411S (gray line), and FH-411T (black line) to show that both FH variants have identical DAA. Abs, absorbance; RU, resonance units.
(reviewed in\textsuperscript{16}). It is currently thought that the amino acid differences between the C-terminal regions of FH and FHR-1 (S1191 and V1197 in FH; L290 and A296 in FHR-1) alter sialic acid recognition,\textsuperscript{17} conferring distinct surface binding specicity to FH and FHR-1 and eliminating the risk of an undesirable competition between them for host tissues. In this context, the changes introduced in our mutant \textit{CFHR1} through gene conversion are particularly relevant because they transform the C-terminal region of the FHR-1 molecule into that of FH, generating a strong competitor for the binding of FH to their ligands on the host cell surfaces. Confirming this hypothesis, functional analysis of the mutant FHR-1 protein using our improved sheep hemolytic assay demonstrated that the mutant FHR-1 strongly competes, in a dose dependent way, the regulation of the complement AP by FH on the cell surface (Figure 5). Importantly, the presence of the mutant FHR-1 protein led to decreased protection of host cells from complement lysis without affecting complement homeostasis (C3 levels) in plasma, a characteristic shared by all complement gene variants associated with aHUS.

Genetic abnormalities associated with aHUS are risk factors modulated by additional genetic and environmental factors. This concurrence of multiple risk factors explains the incomplete penetrance of the disease in carriers of complement genetic abnormalities. A major result of our studies was to find there was a statistically significant difference in the FH plasma levels determined by the nonmutated \textit{CFH-CFHR1} haplotype between patients and asymptomatic carriers of the \textit{CFHR1} mutant. Because, in the context of a competition between FH and mutant FHR-1, the relative amounts of FH and FHR-1 are determinant, these findings provide an explanation for the incomplete penetrance of aHUS among the carriers of the \textit{CFHR1} mutant. Consistent with this, our \textit{ex vivo} assay, using activated HMEC-1 cells, provides experimental evidence that the combination of the mutant FHR-1 and the reduced levels of FH affect complement regulation (Figure 6).

It is well established that environmental factors also contribute to development of aHUS in carriers of predisposing mutations. In this context, it is worth mentioning that the clinical records of these patients illustrate that disease development is associated with known triggers of aHUS and that four of our

### Table 1. Demographic and clinical data of affected carriers of mutant CFHR1

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Origin</th>
<th>Onset (Age)</th>
<th>ESRD (Age)</th>
<th>Recurrences</th>
<th>Transplants (Date)</th>
<th>Current Status</th>
<th>Treatment</th>
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<td>0</td>
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<td>Eculizumab</td>
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\textsuperscript{a}Recurrence when eculizumab dose was reduced.  
\textsuperscript{b}Acute presentation triggered by a Churg–Strauss vasculitis and evolution to ESRD, despite eculizumab treatment.  
\textsuperscript{c}After 1 year of treatment, eculizumab was discontinued in 2015. She became pregnant and had a recurrence in March of 2016, again requiring eculizumab. She gave birth in April of 2016 by Cesarean. Eculizumab was discontinued again August 26, 2016.  
\textsuperscript{d}Onset associated with postpartum and first recurrence with pancreatitis. No clear cause for the second recurrence, which presented with neurologic symptoms.  
\textsuperscript{e}Onset associated with postpartum. The third recurrence was associated with a mesenteric thrombosis.
patients developed aHUS during pregnancy or the postpartum period, a condition particularly relevant to aHUS.18 It would be important to determine whether FHR-1 levels may be actively regulated during pregnancy as it has been reported during infection19 because this will elevate the aHUS risk by increasing the FH/FHR-1 gene competition and trigger the disease onset. In conclusion, we report that the FH levels determined by the nonmutated CFH-CFHR1 haplotype in carriers of the CFHR1 mutant gene who are also Tyr402His heterozygotes (see Concise Methods). Squares and circles are levels of FH determined by the CFH allele in the nonmutated chromosome in patients and unaffected carriers, respectively. Solid squares identify patients treated with eculizumab. (B) Representative experiment of C5b-9 deposition on activated HMEC-1 cells. Levels of C5b-9, measured as fluorescence relative units (see Supplemental Methods), are represented versus levels of FH determined by the nonmutated CFH-CFHR1 haplotype in carriers of the CFHR1 mutant gene who are also Tyr402His heterozygotes. Normal upper limit of C5b-9 deposition in control sera is indicated. Asymptomatic carriers of the mutant FHR-1 protein are depicted with empty circles and aHUS patients, untreated or eculizumab-treated, with empty and solid circles, respectively.

Figure 6. Levels of FH modulate penetrance of aHUS in mutant CFHR1 carriers. (A) FH plasma levels (milligrams per deciliter) determined by the nonmutated CFH-CFHR1 haplotype in carriers of the CFHR1 mutant gene who are also Tyr402His heterozygotes (see Concise Methods). Squares and circles are levels of FH determined by the CFH allele in the nonmutated chromosome in patients and unaffected carriers, respectively. Solid squares identify patients treated with eculizumab. (B) Representative experiment of C5b-9 deposition on activated HMEC-1 cells. Levels of C5b-9, measured as fluorescence relative units (see Supplemental Methods), are represented versus levels of FH determined by the nonmutated CFH-CFHR1 haplotype in carriers of the CFHR1 mutant gene who are also Tyr402His heterozygotes. Normal upper limit of C5b-9 deposition in control sera is indicated. Asymptomatic carriers of the mutant FHR-1 protein are depicted with empty circles and aHUS patients, untreated or eculizumab-treated, with empty and solid circles, respectively.

Measurement of FH and FHR-1 Plasma Levels
FH protein levels were measured from plasma samples by ELISA using in-house mAbs that specifically recognize the FH 402H and FH-402Y alleles13 (see Supplemental Material).

Purification of Native and Mutant FHR-1 and FHR-1/FH Competition Assays
FHR-1, FHR-2, and FHR-5 organize a complex set of hetero-oligomeric molecules26,27 and it is experimentally not possible to isolate FHR-1 from the other FHR proteins without altering the physiologic organization of these complexes. Native and mutant FHR-1 were purified from plasma of normal controls and CFHRII1 mutation carriers by immunoadfinity chromatography as previously described,11 and the capacity of the purified proteins to compete with the activity of FH was assessed in an FH-dependent sheep hemolytic assay using a human serum, which was depleted of 75% of total FH.

C5b-9 Deposition on Endothelial Cells
These experiments were performed using human microvascular endothelial cell line of dermal origin (HMEC-1), essentially as described28 (see Supplemental Material).

Statistical Analyses
Reference range for FH plasma levels was determined using Reference Value Advisor v2.1 as described by Geffré et al.29 Statistical analyses were performed with SPSS software, v21. To compare the FH S411T variant with WT overall regulatory capacity, DAA, FI-cofactor activity, and C-terminal C3b binding, we used t test for independent samples. Variation homogeneity between groups was confirmed by Levene’s test. P<0.05 was considered significant.

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DISCLOSURES

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REFERENCES


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