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Report

Clustering of Missense Mutations in the C-Terminal Region of Factor H in Atypical Hemolytic Uremic Syndrome

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Hemolytic-uremic syndrome (HUS) is a microvasculature disorder leading to microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Most cases of HUS are associated with epidemics of diarrhea caused by verocytotoxin-producing bacteria, but atypical cases of HUS not associated with diarrhea (aHUS) also occur. Early studies describing the association of aHUS with deficiencies of factor H suggested a role for this complement regulator in aHUS. Molecular evidence of factor H involvement in aHUS was first provided by Warwicker et al., who demonstrated that aHUS segregated with the chromosome 1q region containing the factor H gene (*HF1*) and who identified a mutation in *HF1* in a case of familial aHUS with normal levels of factor H. We have performed the mutational screening of the *HF1* gene in a novel series of 13 Spanish patients with aHUS who present normal complement profiles and whose plasma levels of factor H are, with one exception, within the normal range. These studies have resulted in the identification of five novel *HF1* mutations in four of the patients. Allele *HF1Δexon2,* a genomic deletion of exon 2, produces a null *HF1* allele and results in plasma levels of factor H that are 50% of normal. T956M, W1183L, L1189R, and V1197A are missense mutations that alter amino acid residues in the C-terminal portion of factor H, within a region—SCR16–SCR20—that is involved in the binding to solid-phase C3b and to negatively charged cellular structures. This remarkable clustering of mutations in *HF1* suggests that a specific dysfunction in the protection of cellular surfaces by factor H is a major pathogenic condition underlying aHUS.

Hemolytic uremic syndrome (HUS) is a common cause of acute renal failure in children, leading to substantial morbidity and mortality (reviewed in Ruggenenti and Remuzzi 1998). HUS is characterized by a triad of symptoms—microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Typical, epidemic or diarrhea-associated HUS is most common in infants and young children. In the majority of the cases, it is associated with the 0157:H7 strain of *Escherichia coli,* which produces a powerful exotoxin (either verocytotoxin or verotoxin). Other possible triggers of the disease are viruses and neuraminidase-producing microorganisms. The outcome of these patients is usually good, with a complete recovery of renal function within 2 or 3 wk. Atypical, non–diarrhea-associated HUS (aHUS) is most common in older children and adults. The prognosis is poorer than that in typical HUS, with a high (10%–30%) mortality. Renal involvement is a constant feature, and up to 50% of patients may need dialysis. Neurological symptoms are also common, and sequelae may persist for several years. Most cases of aHUS are idiopathic, but predisposing factors such as anticancer drugs, immunosuppressive agents, or oral contraceptives have been reported. aHUS can also develop during either the third trimester of pregnancy or the postpartum period, as well as in association with anti-endothelial antibodies. Although most cases are sporadic, familial cases of aHUS have been described. In these cases, both autosomal dominant (MIM 134370) and recessive (MIM 235400) modes of inheritance have been reported.

A number of observations, including the recurrence of the disease in transplanted individuals and the positive response to plasma exchange in some patients, have pointed to the involvement of a plasma factor in the

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etiology of aHUS. These facts, together with early reports describing the association of aHUS with deficiencies of the plasma protein factor H (Thompson and Winterborn 1981; Pichette et al. 1994; Ohali et al. 1998; Rougier et al. 1998), led to the decisive linkage studies by Warwicker et al. 1998, later confirmed by Ying et al. 1999, revealing that the disease segregates with a chromosome 1q32 region that includes the gene encoding factor H (*HF1*). Furthermore, in one of the families with aHUS that are included in these linkage studies, a missense mutation that had no effect on the levels of factor H was found in the *HF1* gene, strongly supporting a role for factor H in the pathogenesis of aHUS.

Factor H is a plasma protein (molecular mass 155,000 daltons) composed of 20 repetitive units of 60 amino acids called "short consensus repeats" (SCR) (Ripoche et al. 1988), also known as "complement control protein" (also known as "CCP") modules. Factor H is encoded by a single gene located on human chromosome 1q32, within the RCA (regulators of complement activation) gene cluster (Rodríguez de Córdoba et al. 1985; Weis et al. 1987). Five additional factor H–related human plasma proteins have been identified and shown to be encoded by five genes, *FHR1–FHR5,* closely linked to the *HF1* gene (Zipfel and Skerka 1994; Zipfel et al. 1999; Rodríguez de Córdoba et al. 1999; McRae et al. 2000). Factor H controls activation of the alternative pathway of complement in fluid phase and on cellular surfaces. It binds to C3b, accelerates the decay of the alternative-pathway C3-convertase and acts as a cofactor for the factor I–mediated proteolytic inactivation of C3b (Weiler et al. 1976; Whaley and Ruddy 1976; Pangburn et al. 1977). Factor H can also interact with polyanionic molecules (sialic acids or glycosaminoglycans) on certain cellular surfaces, conferring to them resistance to damage as a consequence of complement activation through the alternative pathway (Meri and Pangburn 1990; Pangburn et al. 1991). Complete factor H deficiency leads to a situation of hypocomplementemia that increases the risk of infection by pathogens and that is usually associated with recurrent infections by pyogenic microorganisms. In addition, deficiency of factor H has been associated with systemic lupus ertythematosis, type II membrane-proliferative glomerulonephritis, collagen type III glomerulopathy, and, as indicated above, familial HUS (reviewed in Ault 2000).

To get further insight into the genetic relationship between aHUS and factor H, we have performed mutational screening of *HF1* in a novel series of patients with aHUS. These studies have been facilitated by previous work in our laboratory, related both to the organization of the *HF1/FHR1–FHR5* region of the RCA gene cluster and to the structure of the human *HF1* gene. Figure 1*a* summarizes the organization of the 1q32 genomic region containing the human *HF1* gene and *FHR1–FHR5* genes (for genomic sequences of *HF1* and of *FHR1–FHR5,* see the Entrez Nucleotide Sequence Search Web site). Besides showing the exon/intron organization of the genes within this region, figure 1*a* also illustrates the existence of a number of large genomic duplications (1.2–38 kb long; 85%–97% identity) including different exons of the *HF1/FHR1–FHR5* genes. Since two of these genomic duplications include exons 8/9 and exons 21–23, respectively, of the *HFI* gene, we took special care in the design of the PCR primers used to amplify these *HF1* exons, so that homologous exons in either the *FHR1* gene or the *FHR3* gene would not be amplified. Table 1 shows the primer sequences and PCR conditions that we have used to amplify each of the exons of the *HF1* gene from genomic DNA.

Patients included in these studies were selected on the basis of a single criterion: presenting a clinical history of HUS of non–diarrhea-associated origin. Chronic renal failure and/or unsuccessful renal transplantation led most of them to hemodialysis. Twelve of the patients have no family history of HUS. In only one of the probands, HUS11, the death of the mother, as a consequence of postpartum aHUS, was reported. Our patients have a normal complement profile, and, with the only exception of HUS3, they all present with normal plasma levels of factor H, as determined by ELISA and semiquantitative western blot analyses (table 2). The mutational analysis of the *HF1* gene was performed in genomic DNA from these patients and their available relatives, by both SSCP analysis and automatic DNA sequencing of the *HF1* exons. In several of our patients, *HF1* sequences were also obtained by reverse transcriptase–PCR using total RNA obtained from peripheral blood lymphocytes, as described elsewhere (Sánchez-Corral et al. 2000).

In 4 of the 13 patients with aHUS, we identified *HF1* mutations that were not found in a sample of >100 population-matched *HF1* control chromosomes (fig. 1*b*). Patient HUS2 is heterozygous for a G→T substitution at *HF1* nucleotide position c.3621 in exon 23. This mutation results in a tryptophan-to-leucine change, W1183L, in SCR20. No mutations were found in the other *HF1* chromosome. The W1183L mutation was identified both in genomic DNA and in RNA obtained from the lymphocytes of the patient, but not in either the DNA or the mRNA obtained from his parents, indicating that it is a de novo mutation. Patient HUS3 presented a T→C substitution at nucleotide position c.3663 in exon 23, apparently in homozygosis. This mutation changes the amino acid valine 1197 to alanine in factor H SCR20. No additional mutations to V1197A were found in HUS3. Since levels of factor H in HUS3 were half of normal levels, and since she was also found to be homozygous at c. 257, c.994, c.1277, c.1492, c.1551, c.2089, c.2881, and c.3705 *HF1* polymorphic

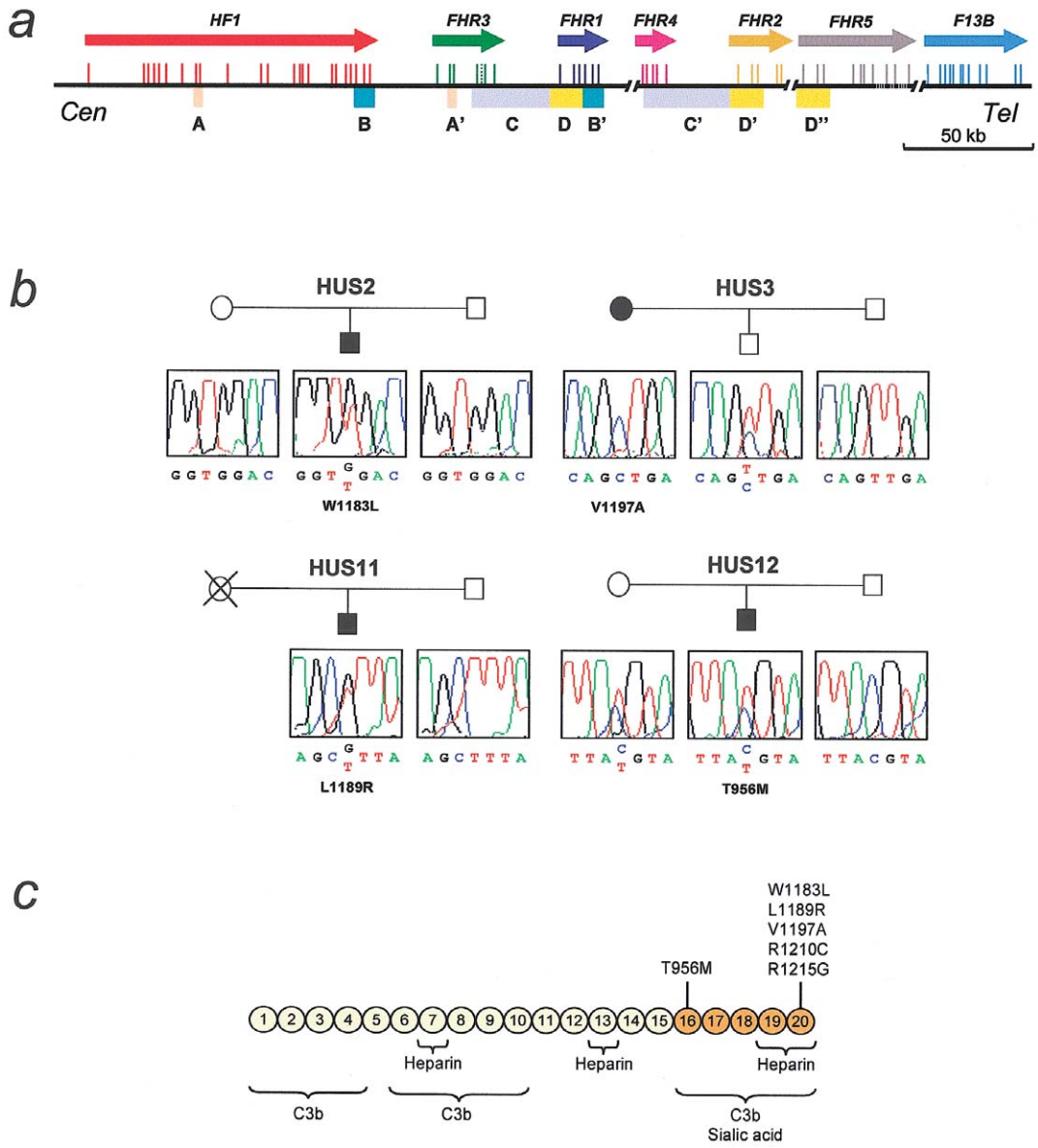

Figure 1 Mutations in factor H in patients with aHUS. *a,* Organization of the 1q32 genomic region containing the human *HF1* gene. Colored arrows indicate the location and transcriptional orientation of the *HF1* gene and the *FHR1–FHR5* genes. For each gene, the exon/intron organization is indicated by vertical bars. Genomic duplications within the region are indicated by colored boxes. These duplications are 1.2–38 kb in size and present a pairwise nucleotide identity of 85%–97%. The exon/intron organization of the *HF1* gene, as well as the nucleotide sequences of the intronic flanking regions, was determined on the basis of data published by Male et al. (2000) and of data generated in our laboratory by PCR analysis and sequencing of PAC clones RPCIP704A14355, RPCIP704L20665, RPCIP704M03650, RPCIP704O14608 (authors' unpublished data). These data and sequence data generated by the Human Genome Project (clones AL049741, AL049744, AL139418, and AL353809) provided the genomic sequence for the *HF1* gene and the *FHR1–FHR5* genes. *HF1* comprises 23 exons and spans >94 kb of genomic DNA. Exon 10 does not contribute to the factor H transcript; it is utilized only in the alternative *HF1* transcript that codes for the factor H–like 1 molecule (also known as "FHL-1") (Estaller et al. 1991). To amplify each of the *HF1* exons present in the factor H transcript, we designed PCR primers to the intronic flanking sequences (table 1). Primers for exons 8/9 and 21–23 were designed to avoid amplification of homologous exons in the *FHR3* gene and the *FHR1* gene, respectively. *b,* Identification of mutations in the factor H gene in four patients with aHUS. The pedigrees of patients HUS2, HUS3, HUS11, and HUS12 are shown, as are the chromatograms corresponding to the DNA sequence surrounding the mutated nucleotide for each patient and family members studied. The positions of nucleotide changes are according to the cDNA sequence reported by Ripoche et al. (1988). The ATG initiation codon is located at nucleotide position c.74. Amino acid numbering includes the signal peptide. *c,* Functional domains and mutations in the factor H molecule. A diagram of the structure of human factor H with the 20 SCR repeats is shown. Functional domains are indicated schematically. The location of the six missense mutations thus far characterized in patients with aHUS (four of whom reported here) is indicated to illustrate that they are clustered within a specific region of factor H, a region that has been reported to be important for the control of C3b deposited on surfaces.

Table 1

PCR Primers for Mutational Analysis of *HF1*

Exon	Protein Region	Primer (5′→3′) Forward	Primer (5′→3′) Reverse	Fragment Size[a] (bp)
1	Signal peptide	GACGTTGTGAACAGAGTTAGCTG	ACTCCTGTGAAAAGCATCATTAG	181
2	SCR1	GTACATTTAAATAGACACTTTATGC	TACACCTAGTTTTCATAAATTTCAC	281
3	SCR2A	CCCACTCCTACATAAAATATATTCC	CCTATTTACTATCTTAATTATAAACC	171
4	SCR2B	TAAACACACATTATGTCAACGTTC	GAGAC TTTAAGATATTTTAATGTAAG	206
5	SCR3	TACATACACATATTTTTCACAATAAAC	GCAAAAATACTAAAACAGTAAGTG	294
6	SCR4	CCTTTAATTTGCAATAAACATTTTGG	TATGTGATAAATTTATAAAGATCCAG	263
7	SCR5	CGGATACTTATTTCTGCATTATCC	AAATTTCAGAATTAAGAAATGGGTC	264
8	SCR6	GTTTATTACAGTAAAATTTCTTTATAC	CTTCGATCTTTGAAAGTTTTATAC	300
9	SCR7	TGAGCAAATTTATGTTTCTCATTTAC	TTAGAAAGACATGAACATGCTAGG	279
11	SCR8	AAAATGTTATTGATCATATGCTTGTC	ACTTTTGTGTATCATCTGGATAATC	284
12	SCR9	GTTTATTAGATGACATTAGAAATGAC	GGAAAACAGATTTATTTTCATTTTG	280
13	SCR10	TTGGCAATGATTAATTATATATTCTC	TCAAAGTTCTAATTCTTATTTCAGC	274
14	SCR11	TATATTGTAAAACAGACAATTTAACC	ATACAAAATACAAAAGTTTTGACAAG	289
15	SCR12	AAAACACATACATCATGTTTTCAC	GTTGTTACAATAAAAATATTAAACTTTG	292
16	SCR13	AGTTGGTTTGATTCCTATCATTTG	ACACACATACCTATTACTTTTCC	277
17	SCR14	ATATTTTTATTTTTTATTTTTTATTATAAC	TATTAACCTCATTTGAAAGAATTATG	290[b]
18	SCR15	GTATTTTATTTGTTTTTAACCCTTTG	ATGAATTCTACTATAAACAGAAATTG	277
19	SCR16	TAAATTTATGAGTTAGTGAAACCTG	TGGTACCACTTACACTTTGAATG	272
20	SCR17	TTTTAAAGATTTGCGGAACAAATAC	CCCACACATTATATAAATAAATTTTG	263
21	SCR18	TTGCTACTCAAAATGAACACTAGG	CCTGCTATACTCCCCCAAAATG	274
22	SCR19	TTGTATTTTGATTTGCTCTCACAAC	GTGAAATATCAGACTCATCACAGA	296
23	SCR20	ATTTGCATACTACTTAATGTTTTATG	AGTTCTGAATAAAGGTGTGCAC	284[c]

[a] Analyzed by SSCP on 8% polyacrylamide gels that were run at room temperature and at 4°C for 20 h, under constant current. All fragments also were gel purified and were sequenced in a ABI-PRISM 377 automatic sequencer. All the PCR reactions were done at an annealing temperature of 57°C in 1.5 mM $MgSO_4$.

[b] A fragment of 477 bp was generated by primers GAAAGTCTATGAGAATACAAGCC and TATTAACCTCATTTGAAAGAATTATG and then was reamplified with the primers given in the table.

[c] A fragment of 396 bp was generated by primers TTTATTCAAATCAATATGATGTTTC and AGTTCTGAATAAAGGTGTGCAC and then was reamplified with the primers given in the table.

sites (to be described elsewhere), we set up experiments to determine whether she carries a genomic deletion in *HF1* in heterozygosis. Segregation analysis of *HF1* polymorphisms in her son demonstrated that she carries a null allele at the c.257 polymorphic site in exon 2 (coding for SCR1), indicating a partial deletion of the *HF1* gene in one of her chromosomes. Although the precise length of the deletion has not been established, we conclude that this deletion results in a null *HF1* allele (*HF1*Δ*exon2*) that is not transcribed into mRNA. Patient HUS3 was, therefore, interpreted to be an *HF1* hemizygote who, in addition, carries the V1197A mutation in her only functional *HF1* allele. Since the nucleotide change involved in the V1197A mutation is one of the two nucleotide differences that distinguish *HF1* exon 23 from *FHR1* exon 6, within the large genomic duplication involving the 3′ end of these two genes (fig. 1*a*), we suggest that the V1197A mutation could have originated via a gene-conversion event between *HF1* and *FHR1*.

DNA sequence analysis of *HF1* demonstrated that a third patient, HUS11, is heterozygous for a T→G substitution at *HF1* nucleotide position c.3639 in exon 23. This mutation results in a leucine-to-arginine change, L1189R, in SCR20. The mutation was not found in the father of the patient, suggesting a maternal origin. However, DNA from the mother, who had died of postpartum HUS, was not available. Finally, in a fourth patient, HUS12, *HF1* mutational analysis demonstrated a C→T substitution in heterozygosis at nucleotide position c.2940 in exon 19. This change results in a threonine-to-methionine change, T956M, in SCR16. Analysis of the DNA from the parents of HUS12 revealed that the mother also carries the T956M mutation in heterozygosis. No mutations in *HF1* were found in the remaining nine patients with aHUS, by either SSCP or DNA sequencing analyses.

Different strategies, involving the use of monoclonal antibodies, enzymatic digestion, or deletion mutagenesis, have allowed the identification and characterization of distinct functional domains in the factor H molecule, although it has not been exactly determined which amino acid residues are responsible for these functions (Alsenz et al. 1985; Gordon et al. 1995; Kühn et al. 1995; Jokiranta et al. 1996; Prodinger et al. 1998). Figure 1*c* depicts schematically some of these functional

Table 2

Complement Profiles and Factor H Levels in Patients with aHUS

Patient (Age [years])	Clinical History	CH50[a]	AP50[b]	C3[c] (mg/100 ml)	C4[c] (mg/100 ml)	Factor H[d] (μg/100 ml)
HUS2 (23)	Chronic renal failure, transplanted twice	Normal	Normal	71.2	30	1,158
HUS3 (53)	Chronic renal failure, hemodialysis	Normal	Low	59.2	26.3	109[e]
HUS5 (4)	Three HUS episodes, neurological symptoms	Normal	Normal	112	15	350
HUS8 (12)	Neurological symptoms	Normal	Normal	96.5	30.3	501
HUS9 (10)	Two HUS episodes, neurological symptoms	Normal	Normal	113	23.5	286
HUS10 (10)	One HUS episode	Normal	Normal	94.1	17	484
HUS11 (12)	Transplanted twice, deceased	Normal	Normal	167	53	1,225
HUS12 (13)	One HUS episode	Normal	Normal	135	27.2	821
HUS13 (10)	Transplanted, hemodialysis	Normal	Normal	86.5	10.6	546
HUS14 (21)	Cyclosporin related, chronic renal failure, transplanted	Normal	Normal	84	24.4	195
HUS15 (17)	Cyclosporin related, chronic renal failure, transplanted	Normal	Normal	96.2	19.5	483
HUS16 (2)	One HUS episode	Normal	Normal	90.8	15.5	1,052
HUS18 (10)	One HUS episode	Normal	Normal	109	15.7	320

[a] Hemolytic assay using antibody-sensitized erythrocytes.

[b] Hemolytic assay using rabbit erythrocytes.

[c] Measured in EDTA-plasma samples and determined by nephelometry (Immage: Backmann). The normal ranges of concentration for C3 and C4 are 77–210 and 14–47 mg/ml, respectively.

[d] Quantitated by a sandwich ELISA using goat polyclonal antibodies (Quidel) and a murine polyclonal antibody (Quidel) and by semiquantitative western blot analyses as described elsewhere (Sánchez-Corral et al. 2000). The range of concentration for factor H was 170–500 μg/ml.

[e] Individual was demonstrated to be heterozygous for an *HF1* null allele.

domains. Factor H has three binding sites for C3b—in SCR1–SCR4, SCR6–SCR10, and SCR16–SCR20. The C3b-binding site in SCR1–SCR4 is the only site essential for the cofactor activity with factor I, but deletion of any of the C3b-binding sites significantly decreases factor H binding to C3b deposited on cellular surfaces (Sharma and Pangburn 1996). In addition, figure 1*c* illustrates that SCR7, SCR13, and SCR16–SCR20 have been found to contain heparin- and sialic acid–binding sites (Pangburn et al. 1991; Blackmore et al. 1996, 1998; Ram et al. 1998).

The T956M, W1183L, L1189R, and V1197A mutations identified in this study alter the amino acid sequence of the factor H molecule without apparently affecting the normal secretion of the protein into plasma (table 2) (for the factor H sequence, see Entrez Nucleotide Sequence Search). One of these mutations lies in SCR16, and the other three in SCR20, the most C-terminal domain of factor H. There are only two other cases of aHUS that have normal levels of factor H and in which mutations in *HF1* have been previously identified, and, most interesting, in both cases the mutations (R1215G and R1210C) also lie in SCR20 (Warwicker et al. 1998; Caprioli et al. 2000). The clustering of missense mutations in the SCR16–SCR20 domains of factor H in patients with aHUS contrasts with the random distribution of (1) *HF1* mutations in individuals with factor H deficiency (Ault et al. 1997; Warwicker et al. 1998; Buddles et al. 2000; Sánchez-Corral et al. 2000) and (2) the *HF1* single-nucleotide polymorphisms thus far described (see above), strongly suggesting a genotype-phenotype correlation in aHUS, which points to the involvement, in the pathogenesis of this disease, of a specific dysfunction in the C-terminal region of factor H.

Although the relative contributions of SCR16 and SCR20 to the functional map of factor H have not been established, it is clear that both SCRs are part of a region that is involved in the binding to solid-phase C3b and to negatively charged cellular structures (such as sialic acid)—and that this region contributes significantly to the capacity of factor H to control activation of the complement system on certain cellular surfaces (Pangburn et al. 2000). Therefore, it is likely that the SCR16–SCR20 mutations found in patients with aHUS result in a defective protection of cellular surfaces by factor H. On the other hand, the T956M, W1183L, L1189R, and V1197A mutations should not alter the cofactor activity of factor H in the fluid phase, which is in agreement with the normal complement activity and C3 levels observed in our patients with aHUS. It is noticeable that in patients HUS2, HUS11, and HUS12 the levels of factor H are considerably elevated. Whether this situation results from an increased transcription of the *HF1* gene or is a consequence of a low turnover of the mutated protein in plasma remains to be determined.

Thus far, two types of mutations in factor H have been observed in patients with aHUS: null *HF1* alleles resulting in decreased levels of factor H and missense mutations in the SCR16–SCR20 region. Interestingly, one of our patients with aHUS (i.e., HUS3) carries both an *HF1* null allele and the V1197A mutation in her only functional

HF1 copy, which suggests that, in some cases, both decreased levels of factor H and mutations in SCR16–SCR20 may be necessary to produce pathologically significant dysfunction of factor H. In this regard, it should be interesting to reevaluate the previously described cases of aHUS that have decreased levels of factor H, in a search for additional *HF1* missense mutations.

As indicated above, nine of our patients with aHUS have no mutations in *HF1* and present normal levels of factor H in plasma. Experiments to further characterize the activity of factor H in the sera of these patients, including the search for potential plasma components that may interfere with the role of factor H in controlling the surface activation of the complement system, would be needed before involvement of factor H can be excluded from the pathogenesis of aHUS in these patients.

Our patients with aHUS who have missense mutations in the SCR16–SCR20 region are *HF1* heterozygotes, which make difficult the purification and analysis of the mutant factor H proteins. Functional characterization of these mutations in recombinant factor H molecules would be needed to confirm that, in patients with aHUS, there is a defective control, by factor H, of the activation of the complement system on cellular surfaces. If this hypothesis is correct, administration of exogenous, functionally active factor H may become a successful therapeutic approach for a significant number of patients with aHUS.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Entrez Nucleotide Sequence Search, http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html (for factor H cDNA sequence [accession number Y00716] and genomic sequences of the *HF1* gene and the *FHR1–FHR5* genes [accession numbers AL049744, AL049741, AL139418, and AL353809])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for autosomal dominant [MIM 134370] and recessive [MIM 235400] HUS)

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