Atypical Hemolytic Uremic Syndrome Associated With Complement Factor H Autoantibodies and CFHR1/CFHR3 Deficiency

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ABSTRACT: Although genetic defect of complement factor H (CFH) is a common cause of atypical hemolytic uremic syndrome (aHUS), development of autoantibodies to CFH (CFH-Ab) is also known to be an acquired cause of aHUS. Recently, a correlation between the development of CFH-Ab and the deficiency of the CFH-related proteins, CFHR1 and CFHR3, was identified. In this study, plasma complement profiles were measured and genetic analysis of the *CFH*, *CFI*, *MCP*, *CFHR1*, and *CFHR3* genes were performed in three female patients diagnosed with aHUS with positive CFH-Ab. Acute stage plasmas of all the three patients revealed low C3, low or low-normal CFH antigenic levels, and high titers of CFH-Ab. All the patients also showed complete plasma CFHR1 deficiency and homozygous genomic deletion of *CFHR1/CFHR3*, but none had *CFH*, *CFI*, or *MCP* mutations. All the patients were treated with plasmapheresis, and two patients required additional immunosuppressive therapy. These patients had a novel subgroup of aHUS characterized by a combination of genetic (a homozygous deletion of *CFHR1/CFHR3*) and acquired (development of CFH-Ab) factors. Patients with this disease may need intensive immunosuppressive therapy in addition to plasmapheresis. Screening for CFH-Ab and the CFHR1/CFHR3 deficiency should be included in the diagnostic tests for patients with aHUS. (*Pediatr Res* 66: 336–340, 2009)

Hemolytic uremic syndrome (HUS) is a clinical syndrome characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (1). In ~90% of childhood cases, HUS follows a prodrome of diarrhea, which is typically caused by verocytotoxin-producing *Escherichia coli* (1). However, atypical cases (aHUS) without preceding diarrhea also occur. These cases show a tendency to relapse, to occur in families, and to have a poor outcome (1,2). Recent advances have shown that aHUS is a disease of complement dysregulation, and that loss-of-function mutations were detected in ~50% of cases in one of the three complement regulator genes: the *CFH* gene encoding complement factor H

(CFH), the *CFI* gene encoding complement factor I, and the *MCP* gene encoding membrane cofactor protein (CD46) (3,4). In addition, few cases of aHUS were associated with gain-of-function mutations in complement activation proteins, complement factor B and C3, as described (5,6). Among these mutations, *CFH* mutations are the most common and account for 14–30% of aHUS cases (3,4,7–10).

In addition to genetic defects associated with aHUS, acquired functional defects of CFH due to the development of anti-CFH autoantibodies (CFH-Ab) have been observed in 6–11% of aHUS cases (11–13). In 2008, Józsi *et al.* (13) isolated a strong correlation between the occurrence of CFH-Ab and the genetic deletion of the *CFHR1* and *CFHR3* genes by identifying 16 patients (11%) with both defects in a cohort of 147 aHUS patients. The *CFHR1* and *CFHR3* genes, which are tightly linked to the *CFH* gene, encode CFH-related protein 1 and 3 (CFHR1 and CFHR3), respectively (14,15).

Here, we report three additional cases of aHUS positive for CFH-Ab with deficiency of CFHR1/CFHR3. Although the mechanism involved in how CFHR1/CFHR3 deficiency leads to the generation of CFH-Ab is currently unknown, it is clinically important to detect CFH-Ab in deciding the treatment option for patients with aHUS. In patients with positive CFH-Ab, plasma infusion or plasmapheresis alone may be insufficient to restore hematologic and renal disturbances and need additional immunosuppressive treatments in the early phase of disease to reduce CFH-Ab production.

MATERIALS AND METHODS

Patients. All the three patients were clinically diagnosed as aHUS, and stool culture and multiplex PCR tests for verotoxin-producing *E. coli* in their stools were negative. This study was approved by the Ethics Committee of Seoul National University Hospital, Seoul, Korea, and informed consent of parents of the each patient was obtained before DNA analysis.

Complement Assays. Plasma concentrations of the complement components C3, C4, and factor B antigens were measured by nephelometry

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(Dade Behring, Paris La Defense, France). CFH and complement factor I antigen concentrations were measured by an ELISA method as described previously (16).

CFH-Ab Assessment. Presence of plasma CFH-Ab was detected by using an ELISA method as previously described (16). Briefly, ELISA plates were coated with 0.3 μg of purified human CFH (Calbiochem, Meudon, France). After washing and blocking free reactive sites with PBS containing 1% BSA, serially diluted sample plasmas were added to the ELISA plates for 1 h at room temperature. After washing, the plates were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-human IgG antibody (Sigma-Aldrich Chemical, Steinheim, Germany). After additional washing, enzymatic activity was revealed using the orthophenyldiamine substrate. Antibody titers were expressed as arbitrary units per mL (AU/mL) calculated as $(S - B) \times 1000/(P - B)$, where S is OD of the test sample diluted at 1:200, B is absorbency of the blank, and P is absorbency of the reference positive plasma diluted (1:200) given an arbitrary titer of 1000 AU/mL.

Western Blot Analysis. Plasma samples of the patients and their available family members were investigated by Western blotting by using an enhanced chemiluminescent kit (Amersham Life Science, Buckinghamshire, UK). Goat polyclonal anti-human CFH (Calbiochem, San Diego, CA) was used as a primary antibody (1:2500 dilution) and a horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as a secondary antibody (1:2500 dilution). The pooled plasma of five healthy volunteers was used as a normal control. There are two differently glycosylated forms of CFHR1 protein, CFHR1 α (one carbohydrate residue) and CFHR1 β (two carbohydrate residues). Western blot analysis of the plasma of the control subjects revealed three strong bands. According to the binding capacity of the primary antibody, which can bind CFH and factor H like protein-1 (CFHL-1, an alternative splicing product of CFH) strongly and also bind CFHR1-5 weakly, the largest band represents CFH (150 kD) and two smaller bands represent CFHR1 α (37 kD) and a mixture of CFHL-1 and CFHR1 β (43 kD). Specific antibodies against CFHR1 and CFHR3 were not available for this study.

Genomic DNA Analysis. The entire coding exons of *CFH*, *MCP*, and *CFI* were amplified from peripheral blood genomic DNA by PCR and directly sequenced in all the three patients. In addition, to screen the homozygous deletion of *CFHR1/CFHR3*, genomic DNA was amplified by PCR using eight specific primer pairs (R1–R8 primer pairs described in Ref. 14) that cover the 100-kb region downstream of the *CFH* gene. We screened 100 control Korean subjects with this method, and the homozygous deletion of *CFHR1/CFHR3* was detected in two subjects.

RESULTS

Patient 1. A 7-y-old girl was admitted to the hospital with abdominal pain, vomiting, gross hematuria, with a blood pressure of 110/64 mm Hg. Laboratory tests revealed microangiopathic hemolytic anemia (Hb 64 g/L, reticulocyte count 11.5%, serum haptoglobin <0.07 g/L), thrombocytopenia ($20 \times 10^9/\text{L}$), and azotemia [serum creatinine 274 μM , estimated GFR by Schwartz formula (17) $23 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$]. Her spot urine protein/creatinine ratio [Pr/Cr, (mg/dL)/(mg/dL)] was 31.9. A stool culture and multiplex PCR test for verotoxin-producing *E. coli* in the stool were both negative. An antinuclear antibody (ANA) test was negative. The hemolytic process was not controlled by 10 sessions of daily plasmapheresis followed by a week of intermittent plasmapheresis (three times per week) and hemodialysis. Therefore, oral corticosteroid treatment ($2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) was started at day 18 and i.v. immunoglobulin (1 g/kg) was administered twice at days 38 and 39. The steroid and immunoglobulin treatment brought clinical improvement without further plasmapheresis. However, aHUS recurred at day 58 with abdominal pain and jaundice (serum total and direct bilirubin levels were 180 and 106 μM). The serum levels of hepatic (alanine aminotransferase 708 U/L) and pancreatic enzymes (amylase 193 U/L) were elevated, and abdominal ultrasonography revealed mildly swollen pancreas and fluid collection with septation in the peripancreatic space. Although the

hepatic and pancreatic symptoms were relieved after 1 wk of supportive care and total restriction of oral intake, the hemolytic process was unresponsive to 1 wk of daily plasmapheresis but the addition of oral azathioprine ($2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) to the treatment regimen resulted in a complete remission with the discontinuation of plasmapheresis. Laboratory tests conducted 5 mo after onset of symptoms revealed no evidence of hemolysis, but moderate renal insufficiency (serum creatinine 115 μM , estimated GFR $23 \text{ mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$) with mild proteinuria. Oral corticosteroid treatment is currently being tapered (Table 1 and Fig. 1A).

Patients 2 and 3. These two patients, when compared with patient 1, showed milder but very similar clinical features and laboratory findings, which were summarized in Fig. 1B, Fig. 1C, and Tables 1 and 2. Both patients had preceding non-bloody diarrhea with stools negative for enterohemorrhagic *E. coli* and verotoxin. In patient 2, stool was positive for norovirus RNA by RTPCR.

Complement and CFH-Ab assay. Table 2 summarizes the plasma complement profiles of these three patients. In all the three patients, the plasma C3 levels were decreased in the acute stage but normalized once in remission. The antigenic levels of CFH were low (Patients 1 and 3) or low normal (patient 2) during the acute stage; however, these levels were also normalized later during follow-up. The initial titers of the CFH-Ab were extremely elevated in all patients. After 3–11 mo of follow-up examinations, the CFH-Ab titers decreased markedly but still above normal.

Western blot and genomic DNA analysis. When compared with three strong bands detected in the plasma of the control subjects, the 37 kD band (CFHR1 α) was completely lacking and the 43 kD band (a mixture of CFHL-1 and CFHR1 β) was weaker in all the three patients, while the 150 kD band (CFH) was comparable. These findings suggest that the patients have a homozygous depletion of CFHR1 in their plasmas. The 37 and

Table 1. The clinical and laboratory findings of the patients

	Patient 1	Patient 2	Patient 3
Age at onset (years)/gender	7/female	9/female	10/female
Family history	—	—	—
Preceding diarrhea	—	+*	+
Initial laboratory findings			
Hemoglobin (g/L)	65	71	56
Reticulocytes (%)	11.5	4.8	7.5
Platelets ($\times 10^9/\text{L}$)	20	18	18
Serum creatinine ($\mu\text{mol/L}$)	274	265	557
eGFR ($\text{mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$)	23	26	13
Extrarenal involvement	Liver/ pancreas	Liver/ pancreas	Liver
Management			
Hemodialysis	+	—	+
Plasma therapy	PE + PI	PE + PI	PI
Immunosuppressants	Pd/AZT/IVIG	Pd	—
Follow-up (months)	5	14	6
No. of recurrence	1	1	1
Final eGFR ($\text{mL} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$)	54	127	112

* Nonbloody diarrhea associated with positive stool norovirus RNA by RT-PCR.

PE, plasmapheresis; PI, fresh frozen plasma infusion; Pd, oral prednisolone; AZT, azathioprine; IVIG, intravenous immunoglobulin G; eGFR, estimated glomerular filtration rate by Schwartz formula.

Figure 1. The clinical courses of three patients (A, patient 1; B, patient 2; C, patient 3). LDH, lactic dehydrogenase; FFP, fresh frozen plasma infusion; PE, plasmapheresis; HD, hemodialysis; Pd, oral prednisolone; AZT, azathioprine; IVIG, i.v. IgG.

43 kD bands of the parents of patient 1 were weaker than those of the controls, which suggests a heterozygous deletion (Fig. 2). Finally, the homozygous deletion of the *CFHR1/CFHR3* genes was confirmed by failure of PCR amplification of the genomic DNA fragments within or near both genes in all the three patients (Fig. 3).

No pathogenic mutations of the *CFH*, *CFI*, and *MCP* genes were detected in any of the patients.

DISCUSSION

CFH, a serum glycoprotein, is composed of 20 consecutive homologous units called complement control protein modules, each comprising ~60 amino acids (18). CFH is a central fluid-phase regulator of the alternative pathway of complement, having both cofactor and decay-accelerating activity (19). The majority (60–70%) of *CFH* mutations is heterozygous missense mutations that cluster in the C-terminal region of CFH, a heparin binding domain (20–22). Such mutants show decreased binding for glycosaminoglycans, endothelial cells, and C3b, the CFH ligand (20,23–25). In addition to genetic defects involving *CFH* or other complement regulator genes, aHUS may also result from an acquired defect such as the development of CFH-Ab (11–13). The binding epitopes of the CFH-Ab are localized to the C-terminal recognition region of CFH, which is a hot spot for genetic mutations (11,12).

The role of CFHRs has also been studied as a cause of aHUS. The genes for CFH and CFHR1–5 are all tightly linked in the regulators of complement activation (RCA) gene cluster at chromosome 1q32. This site is a hot spot for gene conversion events as well as genomic rearrangements (26–28), and a role for gene conversion in generation of *CFH* mutants associated with aHUS was proved (28). In addition, it is recently known that a haplotype containing a deletion of *CFHR1/CFHR3*, which may result from a nonallelic homologous recombination between *CFH* and *CFHR1*, is detected in ~2% of normal population (11), and this polymorphism has been demonstrated to increase the risk of aHUS (29).

Finally, in 2008, Józsi *et al.* (13) identified a strong correlation between the occurrence of CFH-Ab and the absence or reduction of plasma CFHR1/CFHR3. In a large cohort of 147 patients with aHUS, they found 22 (15%) patients had total or near total

Table 2. Complement component profiles of the patients based on acute and convalescent plasmas

	Patient 1		Patient 2		Patient 3		Normal ranges
	Acute	Conv	Acute	Conv	Acute	Conv	
C3	0.54	1.10	0.42	0.91	0.68	1.39	0.70–1.50 g/L
C4	0.26	nd	0.33	nd	0.23	nd	0.10–0.35 g/L
Factor B Ag*	0.16	0.09	0.13	0.10	0.21	0.18	0.09–0.32 g/L
Factor H Ag†	60	103	73	109	58	123	65–140%
Factor I Ag†	104	111	82	91	115	131	70–130%
CFH-Ab‡	>50,000	485	4,900	285	15,600	656	<100

Acute plasma was collected before any plasma therapy in all three patients, and the convalescent plasma was collected at 3 months in patient 1, at 11 months in patient 2, and at 4 months after the onset. The duration between the collection date of convalescent plasmas and the last plasma therapy was 1 month in patient 1, 9 months in patient 2, and 3 months in patient 3.

* Determined by nephelometry.

† Determined by ELISA.

‡ Arbitrary units calculated using a reference positive plasma given an arbitrary titer of 1000 AU/mL.

Conv, convalescent; nd, not determined; Ag, antigenic level.

Figure 2. Western blot analysis of plasma using a commercial polyclonal anti-human CFH antibody. Three bands are seen in lane 1 (pooled control plasmas); CFH of 150 kD, CFH-related protein 1 α (CFHR1 α) of 37 kD, and a mixture of CFH-like protein-1 (FHL-1) and CFHR1 β of 43 kD. The 37 kD band was completely lacking and the 43 kD band was weaker in all the three patients (lanes 2, 5, and 6, respectively), which suggests a complete lack of CFHR1 α /CFHR1 β . The parents of patient 1 (lanes 3 and 4) showed weaker 37 and 43 kD bands than the controls, which suggests a heterozygous deficiency of CFHR1 α /CFHR1 β . Lane 7 is the plasma of a patient with a CFH mutation.

Figure 3. Panel A: A partial map of the human RCA gene cluster on chromosome 1q32 showing four genes (*CFH*, *CFHR3*, *CFHR1*, and *CFHR4*) in tandem arrangement. Exons are indicated as gray vertical lines, and the positions of the two duplicated homologous segments (*B* and *B'*) and the eight primer pairs (R1–R8) are shown. Panel B: Failure to amplify DNA downstream of *CFH* (R2–R6) was noted in all the three patients (Pt 1, Pt 2 and Pt 3), whereas the DNA was amplified in the control subjects (C). These results suggest a homozygous genomic deletion of *CFHR1/CFHR3* due to recombination between segments *B* and *B'*. M, molecular size markers.

CFHR1/CFHR3 deficiency and 16 (11% of the total) of those patients were positive for CFH-Ab. Furthermore, no patients developed CFH-Ab that did not have a CFHR1/CFHR3 deficiency. These findings suggest that CFHR1/CFHR3 deficiency may favor the development of CFH autoantibody. However, the mechanism by which the CFHR1/CFHR3 deficiency leads to the generation of CFH-Ab is currently unknown. Small portion of healthy control subjects and some patients with aHUS have CFHR1/CFHR3 deficiency but not CFH-Ab (11,13). Several clinically unaffected family members of the patients with CFHR1/CFHR3 deficiency and CFH-Ab also showed CFHR1/CFHR3 deficiency without CFH-Ab (13). These findings suggest that the CFHR1/CFHR3 deficiency itself is not sufficient for the development of CFH-Ab and aHUS.

Since three French cases of aHUS associated with CFH-Ab were first described by Dragon-Durey *et al.* in 2005 (12), a total of 24 patients with aHUS associated with CFH-Ab have been reported with the frequency of 6–11% among patients with aHUS (11–13). However, the *CFHR1/CFHR3* genes were studied in 16 patients of the latest study (13) only, and all 16 have deletion of both genes. Our study presents three additional cases with aHUS associated with a CFH-Ab and a homozygous deletion of *CFHR1/CFHR3*.

Almost all the previously reported patients with CFH-Ab positive aHUS as well as our patients were juvenile, suggesting a preferential age in preadolescent children (11–13). Conversely, aHUS associated with *CFH* mutations starts earlier in children, including the neonatal period (4). In addition, the three cases reported here and two of the three French cases (12) showed extra-renal involvements, especially in the liver and pancreas. Conversely, extrarenal involvement in patients with other forms of aHUS has been reported uncommon (4).

In the three French cases (12), the CFH assays were not performed during the acute phase for two of the patients and their plasma CFH activity decreases, whereas their plasma CFH antigenic levels were normal. Conversely, although the CFH activity was not measured in our cases, the CFH antigenic levels were low or low normal in plasma samples collected during the acute stage but were normalized in plasma collected during the convalescent stage. Based on these results, one can speculate that rapid development of a very large amount of CFH-Ab may lead to a transient decrease in circulating CFH. Therefore, a decreased plasma CFH antigenic level in a patient with aHUS does not exclude the possibility of CFH-Ab-associated aHUS.

All the three French patients were ANA positive (12). Similarly, ANA is frequently detected in adult patients with idiopathic thrombotic microangiopathies associated with acquired ADAMST-13 deficiency (30). Conversely, none of the patients in our study exhibited ANA. However, we currently have no explanation for this difference.

In majority of patients with aHUS with/without known genetic mutations, the illness is preceded by a triggering condition such as a nonspecific infection (3). Indeed, all the three patients in our study as well as the three French cases (12) involved a preceding diarrheal illness and/or gastrointestinal symptoms. Therefore, it is likely that a triggering nonspecific infection alters the immunologic response of the host and leads to the development of autoantibodies. This phenomenon is probably more likely to occur in hosts with a genetic defect of CFHR1/CFHR3.

Genetic diagnosis of patients with aHUS can provide relevant information that can be used to predict survival, renal recovery, and transplant outcome. Overall, patients with *MCP* mutations have a milder form of the disease than those with either *CFH* or *CFI* mutations, and many of these patients recover without plasma therapy (3). In addition, screening for CFH-Ab in aHUS patients would be helpful for planning a therapeutic strategy, as patients with CFH-Ab may require intensive immunosuppressive therapy as well as plasma infusion or plasmapheresis. Plasma therapy, which is the mainstay of treatment of aHUS, can remove circulating CFH-Ab, but such treatment cannot block ongoing production of the autoantibodies. The required intensity of immunosuppressive therapy for individual patients may be

proportional to the degree of CFH-Ab production of each patient. All the three patients reported here and the three French patients (12) had recurrences of aHUS, when plasma therapy was stopped. Most likely, this was due to a rebound of CFH-Ab, which was subsequently prevented by the addition of steroid and/or azathioprine to plasma therapy in four of them. These findings suggest that more aggressive immunosuppressive treatment should be started earlier to prevent this sequel and tapering if this treatment should be guided by serial determination of the antibody titers. In addition, determination of CFH-Ab is also necessary if kidney transplantation is necessary to prevent posttransplant HUS recurrence (31,32).

In conclusion, we present three additional patients with a novel subgroup of aHUS that is characterized by a peculiar combination of genetic (a homozygous deletion of *CFHR1/CFHR3*) and acquired (development of CFH-Ab) factors. For patients with aHUS, determination of CFH-Ab is necessary not only for correct diagnosis but also for planning a therapeutic strategy.

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