Complement Factor H–Related Protein 1 Deficiency and Factor H Antibodies in Pediatric Patients with Atypical Hemolytic Uremic Syndrome

Johannes Hofer,* Andreas R. Janecke,† L.B. Zimmerhackl,* Magdalena Riedl,* Alejandra Rosales,* Thomas Giner,* Gerard Cortina,* Carola J. Haindl,* Barbara Petzelberger,* Miriam Pawlik,* Verena Jeller,* Udo Vester,* Bettina Gadner,§ Michael van Hussen,§ Michael L. Moritz,§ Reinhard Würzner,¶ and Therese Jungraithmayr,* for the German-Austrian HUS Study Group

Summary

Background and objectives This study evaluated the relevance of complement factor H (CFH)–related protein (CFHR) 1 deficiency in pediatric patients with atypical hemolytic uremic syndrome (aHUS) by evaluating both the frequency of deletions in CFHR1 and the presence of complement factor H (CFH) antibodies.

Design, setting, participants, & measurements A total of 116 patients (mainly from central Europe) and 118 healthy blood donors were included from 2001 to 2012. The presence of CFHR1 gene deletions was determined in 90 pediatric patients with aHUS and 118 controls by an easy, fast, and cheap PCR assay; 100 patients with aHUS and 42 controls were tested for CFH antibodies by ELISA. Questionnaires were administered to evaluate the clinical and laboratory data.

Results Homozygous deletion in CFHR1 was detected in 32% of the patients with aHUS tested, compared with 2.5% of controls (P<0.001). CFH antibodies were present in 25% of the patients and none of the controls. CFH antibodies were detected in 82% of patients with homozygous CFHR1 gene deletion and in 6% of patients without. CFH antibody–positive patients with aHUS showed a significantly lower platelet nadir at disease onset and significantly less frequent involvement of the central nervous system than did antibody-negative patients. Antibody-positive patients also received plasma therapy more often.

Conclusion Homozygous deletion in CFHR1 is strongly associated with occurrence of CFH antibodies in pediatric patients with aHUS. However, despite this apparent genetic disease predisposition, it cannot be considered an exclusive cause for aHUS. Initial presentation of Shiga toxin–negative HUS with severe thrombocytopenia and no central nervous system complications in pediatric patients is especially suspicious for CFH antibody aHUS.


Introduction

Hemolytic uremic syndrome (HUS) is a systemic disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Typical HUS is mainly associated with gastrointestinal infections by Shiga toxin–producing bacteria, foremost Escherichia coli O157:H7, and patients usually present with preceding diarrhea (1,2). Typical HUS constitutes approximately 90% of all HUS cases in children and occurs mainly in children 0.5–3 years of age (3).

The less frequent atypical HUS (aHUS) form represents a heterogeneous group of disorders associated with dysregulation of the complement alternative pathway. Prognosis is poor, with high risk of recurrence; about 50% of cases progress to ESRD (4,5).

Atypical HUS can occur in all age groups, with sporadic and familial presentations (6). Among the reported cases, approximately 50% had mutations of the complement regulatory proteins factor H (CFH) (7–12), membrane cofactor protein (13–15), or factor I (16–18); mutations occurred less frequently in factor B (19), C3 (20), and thrombomodulin (21).

Recently, factor H–related protein 1 and 3 (CFHR1/3) gene deletions were implicated in the pathogenesis of aHUS (22). The CFH gene and the genes encoding the five CFHR proteins reside in the centromeric 355-kb segment on chromosome 1, known as the “regulator of complement activation” cluster (23,24). CFHR1–5 show high degrees of sequence identity with CFH, and the secreted protein products of these genes are related in structure (24).

Antibodies against CFH have been reported in patients with aHUS (25–27). These antibodies were shown to induce functional CFH deficiency (25–28) by binding to its C-terminal region and thereby reducing its regulatory function. CFHR1 was shown to neutralize CFH antibodies in patients with aHUS (28). Most recent studies (29–34) established a specific
relationship between CFHR1 deficiency and the generation of antibodies against CFH.

Few descriptive reports on clinical and biologic data and treatments for CFH antibody–positive aHUS have been published (25,26,29–33,35–37). This study sought to evaluate the frequency of deletions in the CFHR1–5 genes and the presence of CFH antibodies in pediatric patients with aHUS and in healthy blood donors. One of the principal questions to be answered was whether a homozygous deletion of the CFHR1 gene is a prerequisite for CFH antibody–positive aHUS. In addition, characteristic clinical and laboratory data were assessed in pediatric patients with CFH antibody–associated aHUS.

Materials and Methods

Study Design, Participants, and Inclusion Criteria

The study was performed according to the Declaration of Helsinki (2000) and was approved by the local ethics committee (Innsbruck Medical University). All participants gave informed consent.

From 2001 to 2012, 116 pediatric patients with aHUS (age <18 years at disease onset) were investigated in our retrospective and prospective multicenter study. Blood specimens and questionnaires from the patients were sent to us by different centers that were seeking help with diagnostic work-up, treatment strategies, and scientific cooperation. Thus, EDTA blood and serum samples from patients and 118 healthy blood donors (recruited from a routine blood drive) were analyzed. Because this was a prospective and retrospective study, some of the data are missing. We could not obtain all information or samples from every patient.

All patients presented with the criteria for diagnosis of HUS: acute anemia, thrombocytopenia, and renal dysfunction. Renal dysfunction was defined by one or both of the following criteria: serum creatinine levels greater than normal values according to age and urine protein-to-creatinine ratio >0.2 g/g. Shiga toxin–associated HUS was excluded by PCR for Shiga toxin or ELISA for serum antibodies against lipopolysaccharides.

Clinical and laboratory data, including results of genetic analysis, were retrospectively acquired by a standardized questionnaire completed by 19 CFH antibody–positive and 54 CFH antibody–negative patients. Questionnaires elicited the following information: description of acute phase with renal impairment; hematologic data; BP development; central nervous system (CNS) and further organ involvement; treatment and investigations toward the cause, including stool and serum investigations for enterohemorrhagic E. coli and Shiga toxin.

CFH antibody titer follow-up was performed prospectively. We had recommended this follow-up for all antibody-positive patients. However, because exact preanalytic treatment and shipment on dry ice are absolutely necessary, only some centers sent us suitable samples for follow-up.

Data were compared with those of a control group of 118 healthy blood donors. Although the control group is significantly older than the pediatric aHUS cohort, this age difference does not influence the frequency of genetic deletions because inborn genetic defects are not generated over the years. Nevertheless, there is indeed an influence on possible CFH-antibody positivity because CFH antibodies are thought to occur as a result of an adequate triggering event. To our knowledge, no healthy individuals have been described with CFH antibodies, and CFH antibodies are thought to be the pathogenic hallmark for aHUS.

CFH Antibody Assessment

CFH antibody titer were determined using an ELISA (25). The antibody titer cutoff was defined as 100 AU/ml. Briefly, ELISA plates were coated with purified human factor H (Calbiochem, Meudon, France). Serum was added at a dilution of 1:50, and detection was performed using goat antihuman IgG, labeled with horseradish peroxidase (both from Sigma-Aldrich).

Genetic Analyses

DNA was extracted from peripheral blood samples using an automated extractor according to the manufacturer’s protocols (GenoM 48; Qiagen, Vienna, Austria). The genomic DNA was analyzed for copy number variation of CFHR1–5 genes by comparative genomic hybridization (CGH) using a customized oligoarray and CGHPRO software and by our PCR technique and was compared with that of healthy controls. CGH analysis has been described in detail elsewhere (38). A homozygous deletion of CFHR1 was additionally confirmed by PCR amplification of part of CFHR1 exon 2 using allele-specific primers, CFHR1 2a,5'-GTTTTGTGTATTTCCTCCCAGCAACA and CFHR1 2b,5'-GAATGACACCTCCATTAAATGACAGA, revealing a 252-bp fragment on agarose-gel electrophoresis.

A 199-bp CFHR2 exon 2 fragment was coamplified using PCR-amplified oligonucleotide primers CFHR2 2a,5'-GTTTTGGTTATTTTCCAGCAACA and 2ar,5'-TGGTGACCAT CTCCTCTCTGC as a control for PCR success and to show that our PCR setting can distinguish between different high homologous sequences within the cluster of sequence related CFHR genes.

We amplified 15–25 ng of genomic DNA in a 25-μl reaction volume that included 1× GoTag PCR buffer (Promega, Mannheim, Germany), 1.5 mM MgCl2, primers at 0.8 mM, deoxyribonucleotide triphosphates at 200 mM (final concentrations), and 0.5 U of GoTag polymerase (Promega). The following PCR conditions were used for all amplifications: initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 20 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes.

Statistical Analyses

Data were calculated using SPSS software, version 15.0, for Windows (SPSS, Inc., Chicago, IL). Chi-squared tests, odds ratios, and 95% confidence intervals (CIs) were used for comparisons of categorical data. When expected values in any of the cells of a contingency table were <5, a Fisher exact test was used. A t test (for normally distributed values) and Mann-Whitney U test (for nonnormally distributed values) were used to compare control and study groups according to independent metric variables. P values <0.05 were considered to represent statistically significant differences.
Results

Patients Characteristics

Overall, 116 patients were included in this study and compared with 118 healthy blood donors. Patients had a mean age ± SD of 5.6±4.8 years at time of disease onset; the mean age in the control group was 40.5±3.5 years at time of blood withdrawal (Supplementary Table 1). However, as detailed in the Materials and Methods section, this difference was not considered essential for the key findings.

PCR and CGH Array Analyses

The presence/absence of CFHR1 exon 2 was tested by PCR to detect homozygous CFHR1 deletion in 90/116 patients (patients in whom DNA was available) and 118 controls (Figure 1). Among the patients and controls, 32.2% (n=29) and 2.5% (n=3), respectively, showed a homozygous deletion (P<0.001).

Twenty-eight of 90 patients and 37/118 controls were analyzed using CGH analysis (Table 1). We selected patients and controls irrespective of their sex and age on the basis of the amount of sufficient DNA available.

In 61% of the patients (17/28), CGH analysis detected CFHR deletions: Forty-three percent (n=12) showed a homozygous deletion (3 of whom had an additional heterozygous CFHR3 gene deletion) and 18% (n=5) showed a heterozygous deletion of CFHR1.

No copy number variation of the CFHR2 and CFHR5 genes were detected. One patient who was homozygous for CFHR1 and heterozygous for CFHR3 gene deletions showed an additional heterozygous deletion of CFHR4.

CFHR gene deletions were detected in 35% (13/37) of the controls using CGH: Eight percent (n=3) showed a homozygous deletion and 27% (n=10) a heterozygous deletion of CFHR1, 8 of these together with a heterozygous CFHR3 deletion.

For the 28 of 90 selected patients and the 37 of 118 controls, PCR results showed a 100% match with the CGH analysis for homozygous CFHR1 deletions (Table 1). This aHUS population is out of Hardy-Weinberg equilibrium (Table 2) for analysis of the frequency of CFHR1 deletions.

CFH Antibody Analyses

Serum samples for CFH antibody analysis were available from 100 patients with aHUS and 42 controls (Figure 1). Twenty-five patients with aHUS (25%) and none of the controls were positive for CFH antibodies (P<0.001).

Of the 74 patients in whom DNA and serum samples were available, 30% (n=22) had a homozygous CFHR1 gene deletion. In 82% (18/22) of these patients, CFH antibodies were detected. One antibody-positive patient with a homozygous CFHR1 deletion was the one with the additional heterozygous deletion of both CFHR3 and CFHR4. Of the remaining 52 patients without homozygous CFHR1 deletion, we identified 3 patients (6%) with CFH antibodies.

Statistical evaluation disclosed a significant association between a given homozygous CFHR1 deletion and CFH antibody positivity (OR, 73; 95% CI, 15–361). Heterozygous CFHR1 gene deletion was not significantly associated with CFH antibody production.

For 8 of 25 CFH antibody-positive patients, genetic screening data were available. One patient showed a heterozygous complement factor I mutation; no mutations were found for any other patient (screening for CFH, membrane cofactor protein, C3, and complement factor I).

Twelve patient samples obtained at disease onset or recurrence showed a significantly higher mean CFH antibody titer than the 13 samples obtained during periods of clinical remission (1342±1458 AU/ml versus 405±116 AU/ml; P=0.03). Likewise, in five patients in whom a titer follow-up was available, the highest CFH antibody titers were always found at disease onset or recurrence (Figure 2).

Clinical and Laboratory Characteristics of Patients Positive and Negative for CFH Antibody

Age at disease onset was significantly greater in the CFH antibody–positive group (mean, 7.9±3.4) than the antibody-negative group (median, 2.2 years [interquartile range, 1.0–5.7 years]) (P<0.001). No differences were found for prodromal gastrointestinal symptoms and respiratory tract infections between the groups (Table 3).

CFH antibody–positive patients had significantly less CNS involvement at disease onset compared with patients without CFH antibodies (P=0.02). No differences were found for oliguria/anuria, arterial hypertension, pancreatic involvement, cardiac involvement, hepatopathia, gastrointestinal tract involvement, or need for dialysis or erythrocyte or platelet infusions.

A higher percentage of CFH antibody-positive patients received plasma therapy in the acute phase compared with CFH antibody–negative patients (P=0.006). This is reflected by a significant difference in patients undergoing plasma exchange but not in patients undergoing plasma infusion (Table 3).

CFH antibody–positive patients show a significantly lower platelet count nadir than CFH antibody–negative patients (P=0.008). No differences were found in peak creatinine, hemoglobin, lactate dehydrogenase, C4, C3, or CFH levels (Table 4).

Discussion

A homozygous CFHR1 gene deletion, accurately assessed by an easy, fast, and cheap PCR assay that reliably replaces CGH analysis for detection of homozygous CFHR1 deletions, was common in our pediatric patients with aHUS and was frequently associated with CFH antibodies. This finding suggests that a homozygous CFHR1 gene deletion is a predisposing factor for CFH antibody production.

The frequency of CFH antibody positivity among patients with aHUS in our cohort (25/100 [25%]) was considerably higher than that in published cohorts (25,29,31) from Dragon-Durey et al. (3/48 [6.3%]), Józsi et al. (5/50 [8.3%]), and Moore et al. (13/142 [9.2%]). This difference in antibody prevalence may correspond to the differences in age distribution within these cohorts because our cohort is the only one focusing on pediatric patients with aHUS.

This aHUS population is out of Hardy-Weinberg equilibrium (Table 2) for analysis of the frequency of CFHR1 deletions. Together with similar observations (31), this finding might hint at an unknown advantage for heterozygotes.
Our study revealed a strong association between the generation of CFH antibodies and the presence of homozygous \textit{CFHR1} deletion. How this genetic background is able to increase the risk for CFH antibody production is unclear. Moore et al. (31) have suggested that deficiency of \textit{CFHR1} may result in a failure of immune tolerance to the homologous region in CFH. Other possibilities, such as cross-reactivity with microbial antigens, have been discussed by Rodríguez de Córdoba (39).

Other investigators have also demonstrated that homozygous \textit{CFHR1} deletions are not universally associated with antibodies against CFH. In our cohort, 86% (18/21) of the analyzed CFH antibody-positive patients had a homozygous deletion of \textit{CFHR1}. This finding corresponds with the cohorts of Józsi et al. (29) (14/18 patients [88%]) and the cohort of Moore et al. (31) (10/13 patients [77%]). On the other hand, a significant autoantibody response to CFH can develop in the presence of normal \textit{CFHR1} (31).
In our cohort, we found three patients positive for CFH antibodies without deletion in CFHR1. The presence of CFH antibodies has not been reported in healthy individuals to date, a result confirmed in this study. Homozygous CFHR1 gene deletions, however, were found in 2.5% of healthy individuals in this study, and may be related to the development of CFH antibodies. Thus, one may speculate that the action of an eventual triggering event in individuals with homozygous CFHR1 gene deletion could play a role in setting off the production of CFH antibodies, leading to aHUS. The initial triggering event leading to the development of aHUS or CFH antibodies is still a matter of speculation, as in other autoantibody-mediated diseases (37). It is not clear whether an infectious agent, some other inflammatory or immunologic stimulus, or another unknown factor leads to complement activation.

Atypical HUS is diagnosed in 1 of 10 patients with HUS (40). Although the incidence of complement associated aHUS is not known, one can estimate that the incidence of aHUS is about 1–2×10^-6 (41). The prevalence of homozygous CFHR1 deletion in our population of healthy controls was 2.5%. According to our data, one can calculate the relation between patients with aHUS patients who have a homozygous CFHR1 deletion and healthy individuals with the same deletion to be 1:10^5 (Figure 3). Thus, from the frequency of homozygous CFHR1 deletion in the normal population, a dominant causative link is unlikely. However, a homozygous CFHR1 deletion in aHUS may be a marker for a different genetic background predisposing to aHUS or may be a cofactor.

Knowledge of an asymptomatic individual with a CFHR1 homozygous gene deletion could have important clinical implications, especially when in the context of potential living donors for renal transplantation. Should the potential risk of developing aHUS from CFH antibodies, possibly generated in the future in a potential donor with a CFHR1 homozygous gene deletion, preclude him or her from being a donor? The low absolute risk for individuals with this deletion to develop aHUS would make their exclusion from being a donor questionable. Nevertheless, it could be that the low a priori risk of an individual with a homozygous deletion increases as a consequence of a potential complement activation associated with the transplant procedure. These factors may have to be taken into account when an individual with a homozygous CFHR1 gene deletion is being considered as a kidney donor. So far, we are

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Table 1. Comparison of PCR and comparative genomic hybridization data from patients and controls in whom both methods were performed

<table>
<thead>
<tr>
<th>CGH Results</th>
<th>PCR Results</th>
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<tbody>
<tr>
<td></td>
<td>Homozygous Deletion of CFHR1(n)</td>
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<td>Patients with aHUS (n=28)</td>
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<tr>
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<td>CFHR1 heterozygous deletion</td>
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<tr>
<td>CFHR1 no deletion</td>
<td>0</td>
</tr>
<tr>
<td>Controls (n=37)</td>
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</tr>
<tr>
<td>CFHR1 heterozygous deletion</td>
<td>0</td>
</tr>
<tr>
<td>CFHR1 no deletion</td>
<td>0</td>
</tr>
</tbody>
</table>

CGH, comparative genomic hybridization; aHUS, atypical hemolytic uremic syndrome.

^aOur PCR analysis cannot distinguish between the absence of a deletion and heterozygous deletion of CFHR1.

^bEight of the controls had a heterozygous deletion of CFHR1 and CFHR3, and two had a heterozygous deletion of CFHR1 only.

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Table 2. Results of comparative genomic hybridization analysis in comparison with expected frequencies calculated according to Hardy-Weinberg equilibrium

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal Population (%)</th>
<th>Patients with aHUS (%)</th>
<th>P Value^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CFHR1 deletion</td>
<td>64.8</td>
<td>39.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Expected heterozygous CFHR1 deletions calculated according to HW equilibrium</td>
<td>31.4</td>
<td>46.8</td>
<td>0.25</td>
</tr>
<tr>
<td>Observed heterozygous CFHR1 deletions</td>
<td>27.0</td>
<td>17.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Expected homozygous CFHR1 deletions calculated according to HW equilibrium</td>
<td>3.8</td>
<td>13.9</td>
<td>0.08</td>
</tr>
<tr>
<td>Observed homozygous CFHR1 deletions</td>
<td>8.1</td>
<td>42.8</td>
<td>&lt;0.001</td>
</tr>
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</table>

aHUS, atypical hemolytic uremic syndrome; HW, Hardy-Weinberg.

^aP values are calculated with chi-squared test for the normal population compared with patients with aHUS. Calculations were done on the basis of the observed frequency of individuals without CFHR1 deletions in the normal population and the aHUS population. The expected frequencies are calculated using the HW equilibrium on the basis of the given frequency of “no CFHR1 deletion” for patients and controls.
not aware of any case in the literature with de novo aHUS due to CFH antibodies in either donor with a homozygous CFHR1 deletion or transplant recipient.

We report clinical and laboratory data of 19 CFH-antibody positive pediatric HUS patients. The age at disease onset in our cohort (mean age, 7.9 years) was similar to the published age range (37). Age at onset was significantly greater than that in patients with CFH-antibody negative cases (31,37).

Extrarenal complications in CFH antibody–positive aHUS during the first flare of disease were common, although CNS involvement was found in a significantly lower percentage of patients than in CFH antibody–negative patients (11% versus 38%) and than in non–exclusively pediatric CFH antibody–positive population (37).

The presence of CFH antibodies was associated with a significantly lower platelet nadir at disease onset compared with CFH antibody–negative patients, and even lower than described for the nonexclusively pediatric cohort (37). The platelet nadir in our cohort is quite close to the mean platelet nadir in ADAMTS 13 activity–deficient patients with thrombotic thrombocytopenia (42), which may lead to diagnostic difficulties.

In contrast to dialysis, plasma therapy was used significantly more often in patients with CFH antibodies, but antibody positivity was known for only 5 of 18 antibody-positive patients receiving plasma therapy during the first 4 weeks after disease onset. This fact could reflect the high response rate of this disease entity to plasma treatment at disease onset (43) or a more pronounced plasma dependency during first disease flare.

None of our antibody-positive patients with aHUS received eculizumab at the first disease flare; even today this decision may be justified because aggressive plasmapheresis, followed by maintenance therapy with immunosuppression,
appears to be a good treatment option for patients with CFH antibody–associated aHUS (25,26,29–33,35–37).

CFH antibody titers were significantly higher during disease activity than during remission but may increase again when an adequate triggering event is present. Thus, repeated measurements in these patients are recommended to recognize a possible recurrence as early as possible. Because of a high variability among patients, CFH antibody titers can be interpreted only individually.

In conclusion, patients with aHUS and a homozygous CFHR1 gene deletion frequently have CFH antibodies, and the latter are nearly exclusively found in these patients.
confirming that homozygous CFHR1 gene deletion is related to antibody production. However, this gene deletion on its own is clearly not sufficient for the development of aHUS. Initial presentation of Shiga toxin–negative HUS with severe thrombocytopenia and absent CNS complications in 6- to 10-year-old patients is especially suspicious for CFH antibody aHUS.

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Disclosures
None.

References