Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries

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Background: The aetiology of atypical haemolytic uraemic syndrome (aHUS) is, in contrast to classical, Shiga-like toxin induced HUS in children, largely unknown. Deficiency of human complement factor H and familial occurrence led to identification of the factor H gene (FH1) as the susceptibility gene, but the frequency and relevance of FH1 mutations are unknown.

Methods: We established a German registry for aHUS and analysed in all patients and 100 controls the complete FH1 gene by single strand confirmational polymorphism and DNA sequencing. In addition, complement C3 and factor H serum levels were assayed. Demographic data at onset of aHUS and follow up were compared for the mutation positive and negative groups.

Results: Of 111 patients with aHUS (68 female, 43 male, mean age 33 years), 14% had FH1 germline mutations, including two of eight patients with familial aHUS. For each of these eight patients, both parents were tested and we were able to trace the mutation for five cases. In the other three cases (one with the mutation 3749 C/T, one with 3200 T/C, and one with 3566+1 G/A), we could not detect the mutation in either parent, although paternity was proven by genetic fingerprinting, suggesting that these subjects have new mutations. C3 was decreased in five mutation carriers but also in two non-carriers, and factor H was decreased in none of the carriers, but elevated in six carriers and 15 non-carriers. Clinical parameters including associated medications and diseases, and outcome of aHUS and of post-aHUS kidney transplantation were similar in the mutation positive and negative groups.

Conclusion: FH1 germline mutations occur with considerable frequency in patients with aHUS. Hypocomplementaemia is not regularly associated with a germline mutation, and factor H serum levels can even be elevated. Screening for FH1 mutations contributes to the classification of aHUS.

Haemolytic uraemic syndrome (HUS) is a severe disease frequently leading to end stage renal failure. Clinical features include rapid deterioration of renal function, anaemia negative for Coomb’s test, low platelet count, elevation of lactate dehydrogenase, decreased haptoglobin, and occurrence of fragmented red cells in the blood smear.

Current classification of HUS delineates two major types. Classical HUS occurs almost exclusively in childhood and is caused by bacteria releasing Shiga-like toxins. The disease starts with signs of enteritis, generally initiated by Escherichia coli strains, mainly O157. Atypical HUS (aHUS) usually occurs in adults, and has been reported in association with a variety of conditions such as therapeutic drug usage (ovulation inhibitors, immunosuppressive agents), diseases (malignancies, systemic lupus erythematosus) or pregnancy, and after childbirth. Differentiation of classical and aHUS is important for both treatment and outcome, as patients with aHUS require plasmapheresis with replacement by fresh frozen plasma.

Familial occurrence of aHUS is reported in siblings, in a few families with autosomal dominant inheritance and rarely with autosomal recessive transmission. In some families, affected individuals exhibit decreased plasma levels of C3, indicating defective complement control, and suggesting a role of complement regulators for the disease process.

Factor H is a central fluid phase regulator of complement, and both deficiency and functional inactivation of the plasma protein results in uncontrolled complement activation. The human factor H gene (FH1) is located on the short arm of chromosome 1 (1q32), a region that includes the regulators of complement activation gene cluster. Linkage analysis indicated that FH1, which is located in this cluster, could be a candidate gene for aHUS. Subsequently, independent groups have reported germline mutations in FH1 in additional families, thus corroborating its pathogenetic relevance.

In order to identify the frequency of FH1 mutations in aHUS in general, we established a large registry for aHUS, identified mutations in patients with and without a family history, and compared clinical features of mutation positive and negative patients.

METHODS Registry for aHUS

Nine hundred nephrologists in Germany, Austria, northern and central Switzerland, and northern Italy were contacted in 1998, 2000, and 2002 in order to establish a registry for aHUS in German-speaking countries. Included in this study were all patients for whom blood for further analyses was available. Criteria for HUS were rapid deterioration of renal function, non-immunogenic anaemia, low platelet count and haemolysis, for example, elevated lactate dehydrogenase, decreased haptoglobin, occurrence of fragmentocytes in peripheral blood smear, and thrombotic microangiopathy in renal biopsy. All cases were reevaluated for aHUS by tests for antibodies for the following E coli MTP strains: O157, O26, ...
We used standard primers as reported. 24 Novel primer pairs strand conformation polymorphism (SSCP) of PCR products. product FHL-1, 21 were screened for mutations by single which encodes an exon specific to the alternatively spliced from 10 ml peripheral EDTA blood by standard procedures PolyPhen/)26 was used to predict the likely impact of non-

The PolyPhen server (http://www.bork.embl-heidelberg.de/

Prediction of effects of amino acid substitutions
The PolyPhen server (http://www.bork.cmbi-heidelberg.de/

Factor H ELISA
Factor H concentrations were determined in serum using an ELISA assay.29 The protocol of this study was approved by the ethics committee of the University of Freiburg. All subjects gave informed consent.

For statistical analyses we used Fisher’s two-tailed exact test to compare small groups. For larger groups, the unpaired two-sided χ² test was used. P<0.05 was taken as statistical significance.

RESULTS
Registry
The registry of aHUS for German speaking countries included (as of June 2003) 111 patients with aHUS of non-transplanted kidneys. One case was excluded from the study because of elevated MTP-O157 antibodies. The demographic data are presented in table 1. There were 68 females and 43 males. Age at diagnosis of aHUS was 1 to 78 years (median 32 years); the series included four children aged 1, 2, 2, and 6 years, three adolescents of 13, 14, and 16 years, and four of 17 years. In 81% of the patients aHUS occurred in the decade from 1993 until 2002.

DNA variants of the factor H gene
Analyses of the 111 patients with aHUS revealed 17 germline DNA variants of the factor H gene (table 2). These variants did not occur in the control group (table 3). Consequently, these findings were interpreted as germline mutations. Mutations were observed in 16 patients, and one patient had two mutations. The mutations are located in exons 14, 15, 17, and 19–23, representing at the protein level SCRs 11, 12, 14, and 16–20, respectively. Mutations clustered in the C-terminus of factor H; 82% of the mutations were located in regions coding for SCRs 16 to 20 (exons 19–23). The majority of mutations (13 of 17) were missense, resulting in single amino acid exchanges. Of the additional mutations, one caused a frameshift, one introduced a premature stop at codon 714, one mutation caused a single amino acid deletion at codon 1216, and one mutation affected splicing at nucleotide 3566 (exon 22, SCR 19). All mutations analysed are heterozygous, indicating that each of the individual patients had one intact and one defective allele. All but one of the mutations occurred only once in this series.

For nine of 17 mutation carriers, both parents were tested. Three patients had new (de novo) mutations. The patient

| Table 1 | Demographic data of 98 patients with atypical haemolytic uraemic syndrome |
|---|---|---|---|---|---|
| Patients (n) | 16 | 95 | 111 | 0.074 |
| Age at onset of HUS (median) | 1–59 (29) | 2–78 (32) | 1–78 (32) |
| Sex (M/F) | 7/9 | 36/59 | 43/68 |
| Complement activation parameters | | | | |
| Decreased /increased) serum C3 | GAAATATATTTGTAACTGTTATC (forward primer), | | |
| | | | |
| Decreased /increased) factor H | 0 / (6) | 0 / (15) | 0 / (21) |
| Associated conditions | | | |
| Familial aHUS | 2 | 6 | 8 |
| Drugs (e.g. antithrombotic inhibitors) | 3 (3) | 14 (6) | 17 (9) |
| Gout/unrelated genetic disorders | 1 | 14 | 15 |
| Malignant tumours | 0 | 6 | 6 |
| Signs of infections | 2 | 11 | 13 |
| Other conditions (SLE etc) | 4 | 8 | 12 |
| Treatment and outcome | | | |
| Plasma exchange (FFP alone) | 7 (1) | 69 (2) | 76 (3) |
| Relapse in genuine kidneys | 5 | 23 | 28 |
| Chronic renal failure | 1 | 15 | 16 |
| End stage renal failure | 13 | 69 | 82 |

p Value indicates statistical differences of mutation-positive vs mutation-negative patients. FFP, fresh frozen plasma; SLE, systemic lupus erythematosus.
with two mutations (case 16) inherited the 3135 A/T mutation from the father and the 3701 C/T mutation from the mother. The G3007T mutation (case 4) was transmitted from the father and the 3701 C/T mutation from the mother. The G3007T mutation (case 4), who also had aHUS, revealed the ancestry had aHUS and none had impairment of renal function. Genetic testing of the sister of the patient with the 3007 G/T mutation (case 4), who also had aHUS, revealed the same mutation.

The FHI gene analyses of the 100 healthy controls revealed ten DNA sequence variants. Five variants (994 C/A, 1492 G/A, 2089 A/G, 2707 C/T, 3211 C/T) have no consequence at the amino acid level (silent mutations) and were therefore interpreted as polymorphisms. Four additional DNA sequence variants, which cause an amino acid exchange (62 Val→Ile, 402 His→Tyr, 936 Glu→Asp, 1050 Asn→Tyr), have been reported previously as polymorphisms.8,12 One novel DNA variant, which also results in an exchange of a single amino acid (990 Gln→Asp) was found in two controls and three patients of this series (table 3).

**Table 3** DNA variants of the factor H gene occurring in controls and patients of this series

<table>
<thead>
<tr>
<th>Polymorphism [cDNA nucleotide]</th>
<th>Exon</th>
<th>Consequence (amino acid)</th>
<th>SCR</th>
<th>Healthy probands [n = 100]</th>
<th>Unrelated HUS cases [n = 16]</th>
<th>Mutation [n = 95]</th>
</tr>
</thead>
<tbody>
<tr>
<td>257 G/A**</td>
<td>2</td>
<td>62 Val→Ile</td>
<td>1</td>
<td>0*/<em>4</em>*</td>
<td>0*/0**</td>
<td>0*/5**</td>
</tr>
<tr>
<td>994 C/A/*</td>
<td>7</td>
<td>307 Ala→Ala</td>
<td>5</td>
<td>0*/0**</td>
<td>5*/1**</td>
<td>43*/53**</td>
</tr>
<tr>
<td>1277 C/T/*</td>
<td>9</td>
<td>402 His→Tyr</td>
<td>7</td>
<td>4*/39**</td>
<td>5*/21**</td>
<td>44*/46**</td>
</tr>
<tr>
<td>1492 G/A**</td>
<td>11</td>
<td>473 Ala→Ala</td>
<td>8</td>
<td>0*/<em>0</em>*</td>
<td>0*/0**</td>
<td>0*/0**</td>
</tr>
<tr>
<td>2089 A/G**</td>
<td>14</td>
<td>672 Glu→Gln</td>
<td>11</td>
<td>19*/2**</td>
<td>10*/4**</td>
<td>38*/11**</td>
</tr>
<tr>
<td>2707 C/T/*</td>
<td>18</td>
<td>878 His→His</td>
<td>15</td>
<td>0*/0**</td>
<td>0*/0**</td>
<td>17*/0**</td>
</tr>
<tr>
<td>2881 G/T**</td>
<td>19</td>
<td>936 Glu→Asp</td>
<td>16</td>
<td>17*/3**</td>
<td>20*/5**</td>
<td>39*/12**</td>
</tr>
<tr>
<td>2923 G/T*</td>
<td>19</td>
<td>950 Gln→His</td>
<td>16</td>
<td>2*/0**</td>
<td>0*/0**</td>
<td>2*/0**</td>
</tr>
<tr>
<td>3211 C/T*</td>
<td>21</td>
<td>1046 Thr→Thr</td>
<td>18</td>
<td>0*/0**</td>
<td>0*/0**</td>
<td>2*/0**</td>
</tr>
<tr>
<td>3221 A/T*</td>
<td>21</td>
<td>1050 Asn→Tyr</td>
<td>18</td>
<td>6*/0**</td>
<td>0*/0**</td>
<td>2*/0**</td>
</tr>
</tbody>
</table>

*Heterozygous; **homozygous; *PolyPhen prediction of effect of amino acid substitution.

The variant FHI c.2923 G/T has not been reported as a polymorphism in other series so far and may therefore be associated with a risk for HUS.

**Predicted effects of amino acid substitutions**

PolyPhen is an automated tool for predicting the likely impact of an amino acid substitution on protein structure and function.13 Mutations observed in controls (table 3) were all predicted to be “benign” or only “possibly damaging”, whereas the majority of substitutions in patients were classified as “probably damaging” (table 2).

**Table 4** Kidney transplantations in 36 patients of this series with atypical HUS and non-surgical complications

<table>
<thead>
<tr>
<th>Patients [n]</th>
<th>Mutation positive*</th>
<th>Mutation negative</th>
<th>Total</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organs</td>
<td>10</td>
<td>37</td>
<td>47</td>
<td>0.375</td>
</tr>
<tr>
<td>Living donors</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td>0.409</td>
</tr>
<tr>
<td>Lost organs in first year</td>
<td>8</td>
<td>20</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>after transplantation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relapse and acute rejection</td>
<td>8</td>
<td>19</td>
<td>27</td>
<td>0.322</td>
</tr>
</tbody>
</table>
C3 and factor H serum levels

Serum samples were obtained from 106 of the 111 patients with aHUS. C3 analyses revealed decreased levels in seven patients (five with an FH1 mutation and two without an FH1 mutation) and high levels in 15 patients (one with and 14 without an FH1 mutation). Factor H measurement resulted in elevated levels in six mutation-positive and 15 mutation-negative patients, but in none of the patients with decreased levels.

Clinical findings and correlations

The clinical characteristics of the 16 mutation-positive and the 95 mutation-negative patients are presented in tables 1 and 4. Mutation-positive and mutation-negative subjects showed no difference for age or putative causative conditions. In both groups, resulting in end-stage renal failure in 81% and 73%, respectively (13/16 vs 69/95, p = 0.35).

Six mutation-positive and 29 mutation-negative patients underwent kidney transplantation, with repeated transplantations in three and seven subjects in each group respectively (table 4). From the total of 47 transplanted organs, nine were from living donors; one in the mutation-positive group and eight in the mutation-negative group. Relapse of aHUS and acute rejections occurred in eight mutation positive and 19 mutation negative recipients. Graft loss within the first year due to non-surgical reasons occurred in eight organs (80%) of mutation positive and 20 organs (54%) of mutation negative recipients. Statistically, outcome of kidney transplantation did not differ between the two groups (table 4).

Familial aHUS was present in eight patients. In six of these patients, pedigree analysis revealed evidence for autosomal dominant transmission. In two patients, mutations were one paternal and one maternal allele. All affected family members were carriers. One patient had two mutations on the same allele. In another patient, one mutation was on the maternal allele and the other on the paternal allele.
dominant transmission with variable penetrance, whereas two brothers in the seventh family and two sisters in the eighth were affected, leaving the mode of transmission open to debate. The latter family was the only family with an FH1 germline mutation detected in this study (table 2). In another index case, the father was on dialysis, and was not available for genetic testing.

**DISCUSSION**

We established a registry of German speaking countries, comprising 111 patients from Germany, Austria, Switzerland, and southern Tyrolia/Italy for a systematic clinical and molecular genetic evaluation of patients with atypical HUS (aHUS). (HUS not induced by Shiga-like toxin.)

Based on this registry, we analysed the complete FH1 gene, a candidate susceptibility gene for aHUS,

and found FH1 mutations in 13% of the patients. With this approach, we identified 17 new disease-associated FH1 mutations. One patient had two mutations, and only one of the detected mutations occurred twice. Thirteen of the mutations are novel, and only three mutations have been reported previously (tables 2 and 5). The identified mutations are spread over eight exons, and the reported mutations involve an additional two exons (fig 1). This distribution suggests that the entire FH1 gene should be evaluated for genetic classification of aHUS. The relevance of our findings is sustained based on a parallel search of the FH1 gene in blood samples of 100 controls.

Two of our 16 mutation-positive patients had a relative affected by aHUS, indicating familial cases. From the literature a minority of 25% had a family history for aHUS (table 5). In most families only siblings are affected by aHUS, indicating familial cases. From the literature, most mutations occurred twice.

Tracing the mutations back to the former generation was possible in six of our patients and five reported cases (table 5), but none of the ancestors had episodes of an aHUS. This can be explained in two ways, either as autosomal-dominant transmission with reduced penetrance, or as a recessive trait. The latter has been confirmed by molecular genetic and clinical data of one reported family, (table 5). A similar scenario is likely for our patient 15 who exhibited two mutations, one inherited from the father, the other from the mother. Most mutations (76%) in this registry represent single amino acid changes, and the remainder introduce premature stop codons or cause a deletion of an amino acid in the factor H protein. The fraction of single amino acid mutations is similar to that reported in the literature (72%). These missense mutations may affect secretion of the protein as well as biological function. Recent analyses have begun to unravel the precise function of the C-terminus of factor H and the impact of individual mutations on protein function. 27 28 Both a heparin and a C3b binding site have been considered key features of hereditary aHUS. 19 24 33 Such conditions, however, are rarely reported in cases with proven FH1 mutations. Factor H levels may even be elevated (table 5). Data from our registry show normal C3 levels in 10/16 cases and elevated serum factor H in 6/16 cases (table 2). The frequency of high factor H levels indicates upregulation of factor H synthesis.

This registry allows, for the first time, comparisons of family history, including pedigree analysis of FH1 mutation-positive patients with aHUS and FH1 mutation-negative patients with aHUS (tables 2 and 4). Remarkably, we were able to detect an FH1 mutation in only two of eight patients with familial aHUS. This result corresponds well with another study revealing a 2/19 detection rate. 24 These data suggest the existence of at least one other, so far undetected, susceptibility gene for aHUS.

Associated factors that are considered to trigger aHUS, such as exposure to drugs, the contraceptive pill, pregnancy, and malignancies or non-*E coli* induced infections, occurred with similar frequency in both groups. Again, outcome, including relapse rates in non-transplanted kidneys, did not differ statistically between the groups, with end stage renal failure in 81% of the mutation-positive and 73% in the mutation-negative patients. Thirty-six patients with aHUS underwent kidney transplantation (table 4). Of the 47 transplanted kidneys, the number of grafts lost due to nonsurgical reasons within the first year was not significantly different in the mutation-positive and the mutation-negative group. These data characterise the poor prognosis of aHUS as similarly shown by others. 19 20 21

Regarding the 87% of cases in which we have been unable to identify an FH1 mutation, it may be speculated that other complement regulators such as C4BP, MCP, DAF, and CR1 might be involved in the disease process. In summary, this registry-based study demonstrates that germline mutations of the FH1 gene are relatively frequent in patients with aHUS, and demonstrate a clear role of this immune regulator for the development of the disease. Further biochemical and functional studies of both normal and mutated factor H protein will provide deeper insights into the pathogenesis of aHUS.

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