The role of the regulation of complement alternative pathway in hemolytic uremic syndrome

PhD thesis

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1. Introduction

Hemolytic uremic syndrome (HUS) is the main cause of the acute renal diseases in childhood, related to microthrombi-formation due to the damage of the endothelial cell layer of renal capillaries and small vessels, leading to intravascular hemolysis. Accordingly, the main symptoms of the disease are hemolytic anemia, severe thrombocytopenia and acute renal failure.

The classical form of the disease is typical or diarrhea-associated HUS with a relatively good prognosis, developing due to infections with Shiga-like toxin producing bacteria. The pneumococcal-associated form of HUS (pHUS) is an uncommon complication of invasive pneumococcal disease (IPD). Pathogenesis of pHUS is related to neuraminidase production of *Streptococcus pneumoniae*. Although neuraminidase activity can be present in ~50% of IPD patients, only a minority (0.4-0.6%) of these cases progress to pHUS. The atypical form of HUS (aHUS) occurs in about 10% of the cases, associated with severe outcome, frequent relapses, and high risk of developing end-stage renal disease (ESRD). Pathogenesis of aHUS is related to dysregulation of the complement alternative pathway. It may be caused by autoantibodies against complement factor H, furthermore, genetic variations can be identified in the genes encoding complement components and regulatory proteins of the alternative pathway. Mutations associated with the disease can be loss-of-function mutations in the genes of the regulatory proteins (complement factor H (*CFH*), membrane cofactor protein (MCP, *CD46*), complement factor I (*CFI*) and thrombomodulin (*THBD*)) or gain-of-

function mutations in the genes encoding complement components C3 (*C3*) and factor B (*CFB*), which can cause the dysregulation of the complement alternative pathway and lead to the development of aHUS. Mutations may be identified in about 50-70% of aHUS patients, most of these occur in heterozygous form and show incomplete penetrance (~50%) thus, there are asymptomatic carriers among the family members of patients. In addition to mutations, other genetic and environmental factors have a role in the development of the disease, such as the H3 haplotype of *CFH* and the MCP_{GGAAC} haplotype of *CD46*, which occur more frequently in aHUS patients than in control populations.

Traditionally, first-line treatment of aHUS is plasmatherapy, which removes mutant proteins and autoantibodies, but it does not inhibit the activation of the complement system, thus it does not prevent tissue damage. In the past few years new perspectives have opened in the management of aHUS with the advent of eculizumab. This humanized monoclonal anti-C5 antibody inhibits C5, thereby preventing the generation of the terminal complement complex C5b-9.

2. Objectives

1) With our case-series study we aimed to investigate the complex ethiology of aHUS in patients recorded in our laboratory between 2008 and 2014, thus our first objective was to analyze the clinical characteristics and laboratory parameters as well as the outcome of the disease depending on etiology, treatment and age at onset.

2) In spite of diagnostic capabilities of molecular genetic methods, the genetic background has not been known in most of the Hungarian HUS patients. Thus, our second aim was to optimize DNA sequencing methods of the whole coding region of genes *CFH, CFI, CD46, C3, CFB* and *THBD*, and to investigate the genetic risk of aHUS patients.

3) In the past years, increasing number of lists of newly identified genetic alterations have published, but the functional role of the mutations is rarely known. Therefore, the next aim was to investigate the functional effect of *CFH* mutations identified in our patients on protein level and function.

4) H3 haplotype of *CFH* occurs more frequently in aHUS patients than in healthy control populations, however, the mechanisms which contribute to the disease development are not exactly known. Therefore we aimed to investigate whether the H3 haplotype shows any relationship with serum concentration of factor H.

5) The pathogenetic role of neuraminidase-producing *Streptococcus pneumoniae* in the pathogenesis of pHUS is well studied. Since only a small percentage of invasive pneumococcal disease progresses to pHUS, it may be assumed that host risk factors also can contribute to the development of the disease, but this is less studied. Our next aim was to perform the detailed complement and genetic analysis of pHUS patients.

3. Methods

We studied blood samples from patients and family members with clinical diagnosis of HUS, obtained between 2008 and 2014. 29 patients from 27 families with atypical HUS and 5 patients from 5 families with pneumococcus-associated HUS were enrolled. The control population consisted of 210 healthy, unrelated Caucasian subjects.

DNA isolation from mononuclear cells was carried out by salting-out procedure, DNA concentration was determined by spectrophotometry. Primers used for the PCR amplification were designed by Primer Premier program or according to the literature, the amount of PCR products was determined by agarosegel electrophoresis. Primers and dNTP were removed from the PCR products by applying ExonucleaseI and alkaline phosphatase enzymes, thereafter the PCR products were diluted and sequencing reagents were added. After the sequencing reaction, products were purified by ethanol precipitation then capillary electrophoresis was performed following dissolution and denaturation of the products. PCR-RFLP and real-time PCR were used for genotyping polymorphisms of *CFH*. To detect copy number variations, multiplex ligation-dependent probe amplification was performed in the chromosomal regions of *CFH, CFHR1, CFHR2, CFHR3, CFHR5, CFI* and *CD46* genes.

Levels of C4, factor B and factor I were determined by radial immunodiffusion, whereas the concentration of C3 were measured by immunoturbidimetry. Total activity of the classical pathway was measured by a hemolytic method, and a commercial ELISA kit was used

to determine the total activity of alternative pathway. Level of factor H as well as autoantibodies against factor H were measured by in-house ELISA assays. ADAMTS13 activity was measured based on the fluorescence resulting from the cleavage of FRET-VWF-73 fluorescent quenching substrate. To test the effect of *CFH* mutations on the protein level, allele-specific ELISA was carried out using allele-specific antibodies in individuals heterozygous for the Y402H polymorphism. To test the effect of *CFH* mutations on the regulatory function, factor H hemolytic test was performed.

Statistical analyses were performed using Prism 6 software. Results of the hemolytic test were compared to controls with Dunnett's test following parametric one-way ANOVA. Allele-specific protein levels of H3 and non-H3 carriers were compared using Mann-Whitney U-test. Results were considered statistically significant when p-value was <0.05. Linkage disequilibrium between polymorphisms of *CD46* was calculated using Haploview 4.2 software based on data of the International HapMap Project (www.hapmap.org). *In silico* prediction of potential functional impact of novel mutations was performed using PolyPhen, SIFT, PROVEAN and MutationTaster online software.

4. Results

4.1. Clinical and laboratory parameters of aHUS patients

First appearance of the disease ranged from 1 day to 60 years (median 13.5 years), typically occurred in childhood (53.3%), in most patients already under the age of 2 (30%). The other typical age at onset was the young adulthood, 36.7% of patients was 19-30 years old at the first episode. Similarly to previously published cohorts, the outcome of the disease was worse in these patients: currently only 37.93% of them are in complete remission without renal sequalea, 41.38% of patients needs long-term dialysis due to ESRD, and 20.69% were deceased. Patients under eculizumab treatment had better prognosis, although only a few patients have received targeted complement-therapy to date.Mortality rate was the highest in children under 2 years of age (66.67%), corresponding to data of the previously published case series. Based on our observations these patients constitute a specific group of aHUS patients with rapid disease progression and high mortality, in whom plasma therapy may not be feasible.

Complement profile of aHUS patients in acute phase showed increased complement activation and consumption in most cases: low alternative pathway activity could be observed in 69.23% of the available samples, low levels of C3 in 84.62% of samples, low level of factor I in 53.85%, low level of factor H in 69.23%, and low level of factor B in 69.23% of the samples. Most of these alterations normalized in remission, however, in many cases permanently low level of some

components can be observed (low alternative pathway activity in 47.37%, low level of C3 in 63.16%, low level of factor I in 21.05%, low level of factor H in 15.79% and low level of factor B in 26.32%) which indicates the presence of mutation(s) in components of the alternative pathway.

4.2. Genetic analysis of aHUS patients

Amplification of 66 PCR products and 158 sequencing products were optimized for the sequencing of the whole coding region of *CFH, CFI, CD46, C3, CFB* and *THBD* genes. A total of 27 presumably disease-causing variants were found in 23 patients, while we did not find mutations in 6 patients. A total of 15 previously described and 12 novel mutations were found. 13 mutations were identified in *CFH* in 14 patients, 4 mutations in *CD46* in 4 patients, 4 mutations in *CFI* in 3 patients, 3 mutations in *CFB* in 3 patients and 3 mutations in *C3* in 4 patients. A total of 13 patients (44.8%) carried the H3 risk haplotype of *CFH*, its frequency was 0.259 and 26 patients (89.6%) carried the MCP_{GGAAC} risk haplotype of *CD46*, its frequency was 0.589. Patients often carried one or more risk haplotypes along with mutations and the genetic analysis of family members revealed that in many cases the disease develops in individuals carrying multiple genetic risk factors.

4.3. Investigating the functional effects of factor H mutations

Hemolytic test could be applied in case of 9 mutations. Serum samples of the patients carrying mutations p.W198R, p.Q950H,

p.S1191L+V1197A, p.P1161T and p.R1215Q caused lysis of erythrocytes, suggesting the loss of complement regulatory function, while serum samples of patients with mutations p.S772X, p.T956M, and p.T1216del did not cause lysis. In case of p.R1203W we did not observed lysis in the patient and his mutation-carrier brother, but strong lysis was observed in the mutation-carrier mother.

To test the effect of factor H mutations on the protein level we optimized a previously described allele-specific ELISA method. We could apply this method to investigate 7 mutations. Nearly equal protein level could be measured from the two chromosomes in case of mutations p.T956M, p.S1191L+p.V1197A and p.R1215Q. In the samples of patients carrying mutations p.V609D, p.S722X, p.T1216del, p.C448Y, there was no detectable protein level from one of the chromosomes suggesting very low or zero level of protein expression due to these mutations.

4.4. Effect of the H3 haplotype on the factor H protein level

The allele-specific ELISA method was applied to investigate whether the *CFH* H3 haplotype alone, without the presence of a *CFH* mutation shows any correlation with factor H protein level. A total of 19 H3 haplotype carriers and 61 non-H3 carriers were compared. Significant difference was found between the two groups ($p = 0.0252$), thus less protein can be measured from the H3 carrier chromosomes than from the other chromosomes.

4.5. Complement and genetic analysis of pHUS patients

Activity of the classical and alternative pathways, as well as levels of complement components C3 and C4 were typically decreased in acute disease phase, most of these alterations normalized later in remission. All of the patients were negative for anti-factor H autoantibodies and had moderately decreased ADAMTS13 activity in the acute phase that normalized later in remission.

Heterozygous mutations could be identified in three of the five cases. *CFH* (p.R1149X) leads to the creation of a stop codon expectedly causing premature termination of translation. In accordance with this assumption, the complement factor H level of this patient was below the lower reference limit in remission. In another patient a variation causing p.P50A change in complement factor I was found. This mutation was previously described in two aHUS patients and was shown to result in reduced intracellular and secreted factor I levels *in vitro.* Accordingly, complement factor I level of this patient was below the normal range in acute and remission phase as well. One patient was found to be heterozygous to a *THBD* variation (p.T44I), which has not been previously found in aHUS patients and not reported in healthy controls in international databases. H3 risk haplotype of *CFH* could be identified in one case, while MCP_{GGAAC} risk haplotype was found in 3 cases.

5. Conclusions

1) Infants constitute a specific subgroup of atypical HUS patients, with rapid disease progression and high mortality, in whom plasma therapy may not be feasible. The use of eculizumab should be considered as first-line therapy in these pediatric cases. We would suggest that the treatment should start immediately, once the diagnosis of HUS has been verified and the atypical form is suspected. Urgent investigation of the alternative complement pathway is suitable to stratify patients for eculizumab therapy.

2) Applying the optimized sequencing methods, probably disease causing genetic variations could been identified in ~80% of patients. Based on the genetic analysis of family members – as it was observed in many of our cases - the disease develops in individuals carrying multiple genetic risk factors which may explain the relatively low penetrance of these mutations.

3) Allele-specific ELISA was first used to investigate the functional effect of *CFH* mutations; based on our results this method is suitable to evaluate the pathogenic role of newly identified *CFH* variations. We applied this test along with the factor H hemolytic test. Based on our results a total of five mutations were assumed to cause reduced regulation of the alternative pathway, and four mutations were supposed to cause no or minimal protein expression from the mutation-carrier chromosomes.

4) Applying the allele-specific ELISA we showed that lower factor H protein level can be measured from the H3 haplotype carrier chromosome than from the non-H3 carrier chromosomes. We assume that the H3 haplotype itself can affect the level of the factor H production.

5) We carried out first the thorough investigation of complement profile and genetic analysis of pHUS patients. Based on these observations, we conclude that severe complement dysregulation and consumption, in addition to neuraminidase action, accompany the progress of pHUS and the genetic variations of complement genes may contribute to the development of this complication in a proportion of the affected patients.

6. List of publications

6.1. Publications related to the thesis

Mohlin FC, Nilsson SC, Levart TK, Golubovic E, Rusai K, Müller-Sacherer T, Arbeiter K, Pállinger É, Szarvas N, Csuka D, Szilágyi Á, Villoutreix BO, Prohászka Z, Blom AM (2015) Functional characterization of two novel non-synonymous alterations in CD46 and a Q950H change in factor H found in atypical hemolytic uremic syndrome patients. Mol. Immunol. 65:(2) pp. 367-376.

IF:2.973 (2014)*

Szarvas N, Szilagyi A, Tasic V, Nushi-Stavileci V, Sofijanova A, Gucev Z, Szabo M, Szabo A, Szeifert L, Reusz G, Rusai K, Arbeiter K, Muller T, Prohaszka Z (2014) First-line therapy in atypical hemolytic uremic syndrome: consideration on infants with a poor prognosis. Ital J Pediatr 40:(1) Paper 101. 9 p. IF:1.523 (2014)

Szilágyi Á**, Kiss N**, Bereczki Cs, Tálosi Gy, Rácz K, Túri S, Györke Zs, Simon E, Horváth E, Kelen K, Reusz Gy, Szabó AJ, Tulassay T, Prohászka Z (2013) The role of complement in *Streptococcus pneumoniae*-associated haemolytic uraemic syndrome. Nephrol Dial Transplant 28:(9) pp. 2237-2245.

IF: 3.488 (2013)

** Impact factor of journal concerning to 2015 is not yet available*
***shared first authors*

Cumulated impact factors of the publications related to the dissertation: 7,984

6.2. Publications not related to the thesis

Kiss N, Barabas E, Varnai K, Halasz A, Varga LA, Prohaszka Z, Farkas H, Szilagyi A (2013) Novel duplication in the F12 gene in a patient with recurrent angioedema. Clin. Immunol. 149:(1) pp. 142-145.

IF: 3.992 (2013)

Jankovics F, Henn L, BujnaA, Vilmos P, Kiss N, Erdelyi M (2011) A Functional Genomic Screen Combined with Time-Lapse Microscopy Uncovers a Novel Set of Genes Involved in Dorsal Closure of Drosophila Embryos. PLOS ONE 6:(7)

IF: 4.092 (2011)

Cumulated impact factors of the publications not related to the dissertation: 8,084
Cumulated impact factors: 16,068