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Genetic analysis and functional characterization of novel mutations in a series of patients with atypical hemolytic uremic syndrome

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ABSTRACT

Atypical hemolytic uremic syndrome (aHUS) is a rare disorder caused by dysregulation of the complement alternative pathway, and associated with mutations in genes of complement components and regulators. In the recent years several studies have been published describing these mutations, however, no data is available from the Central and Eastern European region. In this study we present a detailed genetic analysis of our 30 patients, hospitalized with the diagnosis of aHUS in the past 7 years. We analyzed the genetic variants of genes *CFH, CFI, CD46, THBD, CFB* and *C3*; furthermore the possible effect of mutations that may alter the function or level of factor H protein was also investigated. We identified 27 (12 novel and 15 previously described) potentially disease-causing mutations in the candidate genes in 23 patients. Genetic analysis of family members revealed that in most cases the disease develops in individuals with multiple genetic risk factors, which may explain the low penetrance of the mutations. Here we showed that two novel mutations (p.W198R, p.P1161T) and a previously reported one (p.R1215Q) in *CFH* caused impaired regulation as indicated by increased lysis in hemolytic test, while four *CFH* mutations (p.V609D, p.S722X, p.T1216del and p.C448Y) were associated with decreased factor H protein level in serum as determined by allele-specific immunoassay. These results further point to the necessity of complete genetic workup of patients with aHUS and to the importance of functional characterization of novel variations.

1. Introduction

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy characterized by hemolytic anemia, thrombocytopenia and acute renal failure (Loirat and Fremeaux-Bacchi, 2011). In the typical form of HUS the symptoms are caused by infection with Shiga toxin-secreting *Escherichia coli* bacteria, in contrast to atypical HUS (aHUS) in which the pathogenesis is related to dysregulation of the alternative complement pathway, leading to uncontrolled activation of the complement system, resulting in endothelial cell damage and thrombotic microangiopathy (Ruggenenti et al., 2001). The atypical form is less frequent (accounts for approximately 10% of all HUS cases (Noris and Remuzzi, 2009)) and has a worse prognosis with high mortality, high risk of progressing to end-stage renal disease and often follows a relapsing course (Caprioli et al., 2006; Jokiranta et al., 2007; Kavanagh et al., 2006).

In about 50-70% of aHUS patients, mutations can be identified in genes of complement components and regulators such as

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AP, alternative complement pathway; ELISA, enzyme-linked immunosorbent assay; ESRD, end-stage renal disease; CKD, chronic kidney disease; FB, factor B; FH, factor H; FI, factor I; MODS, multiple organ dysfunction syndrome.

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complement factor H (*CFH*), complement factor I (*CFI*), membrane cofactor protein (*CD46*), complement factor B (*CFB*), complement component 3 (*C3*) and thrombomodulin (*THBD*) (Caprioli et al., 2006; Kavanagh and Goodship, 2010; Loirat and Fremeaux-Bacchi, 2011). It should be noted, however, that the penetrance in pedigrees with mutations is incomplete, suggesting that it is altered by other genetic and environmental factors (Kavanagh et al., 2013). Beside mutations, carriage of the H3 haplotype of the *CFH* gene, containing the T allele of rs3753394, G allele of rs3753396 (p.Q672Q) and T allele of rs1065489 (p.E936D) polymorphisms, was reported as a risk factor for developing aHUS in several studies (Caprioli et al., 2003; Fremeaux-Bacchi et al., 2005). Furthermore, the so called MCP_{ggaac} haplotype of the *CD46* gene, comprising G allele of rs2796267, G allele of rs2796268, A allele of rs1962149, A allele of rs859705 and C allele of rs7144 has also been described to be associated with aHUS (Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005). As suggested by previous studies (Bresin et al., 2013; Sullivan et al., 2011), identifying the presence of the *CFH* and *CD46* risk haplotypes may help to evaluate the risk of developing aHUS in unaffected family members carrying a mutation. Presence of factor H autoantibodies (Dragon-Durey et al., 2005) associated with homozygous deletion of the complement factor H-related genes 1 and 3 (*CFHR1* and *CFHR3*) (Jozsi et al., 2008) has also been described as a predisposing factor for aHUS. Recently, it has been found that variations of diacyl-glycerol kinase epsilon (*DGKE*) could be responsible for non-complement-mediated forms of aHUS (Lemaire et al., 2013).

In this study we aimed to comprehensively describe and characterize genetic variations observed in aHUS patients and in their family members. Furthermore, we aimed to evaluate the possible functional effect of the novel variations in *CFH* gene, for this we applied allele-specific factor H immunoassay, factor H hemolytic test and *in silico* methods.

2. Patients and methods

2.1. Patient selection

Patients with atypical HUS were prospectively enrolled from January 2008 to September 2014. We included patients with clinical diagnosis of aHUS based on the triad of microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure (Noris and Remuzzi, 2005), who received care in Hungary or whose blood samples were sent to Budapest for complement analysis (except for one patient, whose complement profile was determined elsewhere), and for whom genetic workup (see below) was carried out in our laboratory. Exclusion criteria were: HUS after Shiga toxin–producing *E. coli* infection, or HUS in the context of *Streptococcus pneumoniae,* or HUS secondary to concomitant disease (autoimmune, malignant, drug-related secondary forms), or aHUS with anti-factor H autoantibody. A total of 30 patients were finally enrolled in this genetic study, including six patients for whom detailed case histories were previously reported (Mohlin et al., 2015; Szarvas et al., 2014) and 7 patients for whom partial genetic results were previously reported (Reusz et al., 2011). Written approvals for the diagnostic tests and genetic analysis were given by the patients or their parents. Relevant clinical and laboratory data were collected and analyzed subsequently from hospital records, according to protocols approved by the institutional review board of the Semmelweis University of Budapest and the National Ethical Committee (Scientific and Research Ethics Committee of the Medical Research Council).

In order to define the normal range of the allele-specific ELISA in healthy individuals, 210 blood donors (91 males/119 females) were screened by PCR–RFLP before ELISA was carried out.

2.2. Blood sampling

Peripheral blood samples were collected by venipuncture or from central venous catheter. Freshly collected and separated serum and plasma samples from Hungary were shipped in packages on 4 °C as well as EDTA-anticoagulated blood samples for DNA preparation, while aliquots of samples from other countries were shipped on dry ice to the research laboratory. Serum and plasma samples were stored at −80 °C while EDTA-anticoagulated blood samples were stored at −20 °C, until determinations. "Acute" blood samples were collected during hematologically active disease flare, while "remission" samples were collected in hematologic remission, regardless of the renal status.

2.3. Complement analysis

We performed the following determinations during the diagnostic evaluation of patients: functional assessment of the activity of the alternative complement pathway, concentrations of complement C3, complement factors H, B and I, as well as of anti-factor H IgG autoantibodies. Detailed methods have been described earlier (Reti et al., 2012; Szilagyi et al., 2013).

2.4. Molecular genetic analysis

To screen for mutations the whole coding regions of the genes encoding complement factor H (*CFH*; MIM# 134370), factor I (*CFI*; MIM#217030), membrane cofactor protein (*CD46*; MIM#120920), thrombomodulin (*THBD*; MIM#188040), factor B (*CFB*, MIM#138470) and C3 (*C3*, MIM#120700) were analyzed by direct DNA sequencing following PCR amplification. Detailed methods have been published previously (Szilagyi et al., 2013). Primer sequences and PCR conditions are available upon request.

Polymorphic variants were numbered from the A of the ATG translation initiation site as +1. Previously identified missense as well as nonsense and splice site mutations were accepted as pathogenetically relevant variations. Novel missense variants were regarded as mutations if they were not found or found with zero minor allele frequency in international databases (dbSNP (www.ncbi.nlm.nih.gov/snp)), Exome Variant Server (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/) and 1000Genomes Project phase 3 (http://browser.1000genomes.org/index.html)).

2.5. PCR-RFLP

Genotype of *CFH* c.1204C>T (rs1061170; p.Y402H) was determined by PCR-RFLP method (Biro et al., 2006) in control samples and family members, while direct DNA sequencing revealed the genotype of this polymorphism in patients.

2.6. In silico *prediction*

Possible functional effect of novel mutations was predicted *in silico* using online prediction tools, such as PolyPhen (version2) (Adzhubei et al., 2010) (http://genetics.bwh.harvard.edu/pph2/), SIFT (Ng and Henikoff, 2001) (http://siftdna.org/www/Extended_SIFT_chr_coords_submit.html), PROVEAN (Choi et al., 2012) (http://provean.jcvi.org/genome_submit.php) and MutationTaster (Schwarz et al., 2010) (http://mutationtaster.org).

2.7. MLPA

In order to study copy-number alterations of selected complement genes, multiplex ligation-dependent probe amplification (MLPA) was performed with SALSA MLPA probemixes P236-A3 and