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Complement factor H, FHR-3 and FHR-1 variants associate in an extended haplotype conferring increased risk of atypical hemolytic uremic syndrome

Maria E. Bernabéu-Herrero[a,d], Miguel Jiménez-Alcázar[a,1], Jaouad Anter[c,d], Sheila Pinto[c,d], Daniel Sánchez Chinchilla[c,d], Sofía Garrido[b,d], Margarita López-Trascasa[b,d], Santiago Rodríguez de Córdoba[c,d], Pilar Sánchez-Corral[a,d,*]

[a] Unidad de Investigación, Hospital Universitario La Paz-IdiPAZ, Paseo de la Castellana 261, 28046 Madrid, Spain
[b] Unidad de Inmunología, Hospital Universitario La Paz-IdiPAZ, Paseo de la Castellana 261, 28046 Madrid, Spain
[c] Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain
[d] Ciber de Enfermedades Raras (CIBERER), Madrid, Spain

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ABSTRACT

Atypical hemolytic uremic syndrome (aHUS) is a severe thrombotic microangiopathy affecting the renal microvasculature and is associated with complement dysregulation caused by mutations or autoantibodies. Disease penetrance and severity is modulated by inheritance of "risk" polymorphisms in the complement genes *MCP*, *CFH* and *CFHR1*. We describe the prevalence of mutations, the frequency of risk polymorphisms and the occurrence of anti-FH autoantibodies in a Spanish aHUS cohort ($n = 367$). We also report the identification of a polymorphism in *CFHR3* (c.721C>T; rs379370) that is associated with increased risk of aHUS (OR = 1.78; CI 1.22–2.59; $p = 0.002$), and is most frequently included in an extended risk haplotype spanning the *CFH-CFHR3-CFHR1* genes. This extended haplotype integrates polymorphisms in the promoter region of *CFH* and *CFHR3*, and is associated with poorer evolution of renal function and decreased FH levels. The *CFH-CFHR3-CFHR1* aHUS-risk haplotype seems to be the same as was previously associated with protection against meningococcal infections, suggesting that the genetic variability in this region is limited to a few extended haplotypes, each with opposite effects in various human diseases. These results suggest that the combination of quantitative and qualitative variations in the complement proteins encoded by *CFH*, *CFHR3* and *CFHR1* genes is key for the association of these haplotypes with disease.

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; ANOVA, analysis of variance; C3, complement component 3; *C3*, C3 gene; C3G, C3-glomerulopathy; C4, complement component 4; *C4*, C4 gene; CGH, comparative genomic hybridization; *CFH*, complement factor H gene; *CFI*, complement factor I gene; *CFHR1*, complement factor H-related 1 gene; *CFHR3*, complement factor H-related 3 gene; CI, confidence interval; *DGKE*, diacylglycerolkinase epsilon gene; ESRD, end-stage renal disease; FH, factor H; fHbp, factor H-binding protein; FHR, factor H-related; FHR-1, factor H-related protein 1; FHR-2, factor H-related protein 2; FHR-3, factor H-related protein 3; FHR-4, factor H-related protein 4; FHR-5, factor H-related protein 5; FI, factor I; HRP, horseradish peroxidase; HW, Hardy–Weinberg; MCP, membrane cofactor protein; MLPA, multiplex ligation-dependent probe amplification; OR, odds ratio; PBLs, peripheral-blood leukocytes; PCR, polymerase chain reaction; RCA, regulators of complement activation; SCR, short consensus repeat; SNP, single nucleotide polymorphism; STEC, Shiga toxin-producing *E. coli*; Stx, Shiga toxin; *THBD*, thrombomodulin gene; tHUS, typical hemolytic uremic syndrome; UTR, untranslated region.

* Corresponding author at: Unidad de Investigación, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain. Tel.: +34 912071026.
E-mail address: pilar.sanchezcorral@salud.madrid.org (P. Sánchez-Corral).
[1] Present address: Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Hamburg-Eppendorf, Hamburg, Germany.

1. Introduction

Hemolytic uremic syndrome (HUS) is a rare disease clinically defined by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Most cases result from infections with Shiga toxin-producing bacteria, particularly *Escherichia coli O157:H7*, and are accordingly referred to as STEC-HUS, or Stx-HUS. This presentation primarily affects children and generally has a good prognosis, with full hematological and renal recovery within a few weeks. In approximately 5–10% of cases, however, HUS is not directly caused by a bacterial toxin. This atypical form (aHUS) presents in children and adults and has a poor prognosis, with a mortality/end stage renal disease (ESRD) rate of 10–25% in acute episodes, and approximately 50% of the surviving patients developing ESRD in the long term (Noris and Remuzzi, 2009; Loirat and Frémeaux-Bacchi, 2011).

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aHUS is a multifactorial disease in which both environmental and genetic factors concur. Infections, immunosuppressants, antitumoral drugs, oral contraceptives and pregnancy are thought to initiate the pathogenic cascade that can lead to aHUS in genetically susceptible individuals. Approximately 50% of patients with aHUS have mutations or autoantibodies in the alternative pathway of the complement system that amplify the initial endothelial damage and favor HUS development (Sánchez-Corral and Melgosa, 2010; Kavanagh et al., 2013). Thus, single or combined mutations in the complement genes complement factor H (*CFH*), membrane cofactor protein (*MCP*), complement factor I (*CFI*), complement component 3 (*C3*) and complement factor B (*CFB*) have been found in 44% of 795 aHUS patients from 4 European cohorts (Bresin et al., 2013). Other genes that can be mutated in some patients are thrombomodulin (*THBD*) (Delvaeye et al., 2009), which is a cofactor for thrombin, and diacylglycerolkinase epsilon (*DGKE*) (Lemaire et al., 2013), which is an enzyme that catalyzes the conversion of diacylglycerol to phosphatidic acid. On the other hand, anti-factor H (anti-FH) autoantibodies have been observed in 6–11% of pediatric cases and in a few adult cases (Dragon-Durey et al., 2010). Approximately 90% of these patients are homozygous for the genomic deletion of Complement *CFHR1* and *CFHR3* genes, coding for the FH-Related (FHR) proteins FHR-1 and FHR-3, respectively (Zipfel et al., 2007; Józsi et al., 2008; Dragon-Durey et al., 2009; Abarrategui-Garrido et al., 2009; Moore et al., 2010; Noris et al., 2010).

Complement mutations in patients with aHUS generally present as heterozygous, with approximately 50% disease penetrance in mutation carriers. Genetic variants in *CFH* (Caprioli et al., 2003), *MCP* (Esparza-Gordillo et al., 2005) and *CFHR1* (Abarrategui-Garrido et al., 2009) are associated with a higher risk of aHUS, suggesting they are genetic co-predisposing factors. In fact, the *CFHtgtggt* haplotype (known as *CFH(H3)*, Hageman et al., 2005) and the *MCPggaac* haplotype have been shown to increase aHUS penetrance in complement mutation carriers (Bresin et al., 2013; Esparza-Gordillo et al., 2005; Sansbury et al., 2014). The effect of the *CFHR1*B* allele on aHUS penetrance has not been properly addressed. The *CFH*, *CFHR1* and *MCP* genes are located within the regulator of complement activation (RCA) gene cluster on human chromosome 1q32 (Rey-Campos et al., 1988). *CFH* and *CFHR1* are separated by only 72.25 kb, so there is the possibility that the aHUS risk variants *CFH(H3)* and *CFHR1*B* are in linkage disequilibrium. Along these lines, a recent report on a large family with many aHUS cases revealed that all chromosomes with the *CFH(H3)* haplotype also carried the *CFHR1*B* allele (Sansbury et al., 2014).

We have analyzed complement mutations, anti-FH autoantibodies, and *CFH*, *MCP* and *CFHR1* haplotypes in a Spanish cohort of aHUS patients ($n = 367$) to determine their frequency and to establish potential genotype-phenotype relationships with demographic and clinical data. We have also identified another aHUS risk variant in the *CFHR3* gene, which confirms the presence of an extended *CFH-CFHR3-CFHR1* haplotype conferring increased genetic susceptibility to aHUS. A comprehensive genetic and immunological analysis of aHUS patients, and an appropriate understanding of the clinical consequences of the disease-associated genetic variations is becoming essential to anticipate patient evolution and individualize therapeutic strategies.

2. Patients, materials and methods

2.1. Patients and controls

Blood samples from atypical HUS (aHUS, $n = 367$), typical HUS (tHUS, $n = 43$) and C3 glomerulopathy (C3G, $n = 32$) patients were centrifuged to obtain serum and EDTA-plasma and were stored at −80 °C until used. Peripheral-blood leukocytes (PBLs) were used to prepare genomic DNA by standard procedures. Blood samples from patients with aHUS were collected at HUS onset and/or at relapses. All the patients or their relatives gave written informed consent, as approved by the ethical committees from University Hospital "La Paz" or the Biological Research Center. Serum, EDTA-plasma and DNA were also obtained from a total of 92 healthy Spanish adult volunteers as controls.

2.2. Protein studies

Serum or plasma samples from the 367 patients with aHUS were analyzed by Western blot with various sets of polyclonal antibodies recognizing FH and the FHR proteins (Abarrategui-Garrido et al., 2009) to identify homozygous FH/FHR deficiencies or abnormal bands. These samples were also checked for the presence of circulating anti-factor H autoantibodies by using the original ELISA test (Dragon-Durey et al., 2005).

Levels of FH in plasma samples were measured by a sandwich ELISA, using "in house" polyclonal and monoclonal (moAb214) antibodies. The epitope recognized by moAb214 was localized to SCRs 10–11 of FH by Western-blot, using recombinant fragments of FH expressed in *Pichia pastoris*; this monoclonal antibody does not cross react with any of the FHRs. Plates with 96 wells were coated with 100 μL of the "in house" polyclonal rabbit anti-human FH antibody diluted in 0.1 M $NaHCO_3$, pH 9.5, and incubated at 4 °C overnight. The plates were washed and blocked with 50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20 and 1% BSA for 1 h at room temperature. Two serial dilutions (1:3000, 1:6000) of each plasma sample were added, and incubated for 1 h. FH binding was then detected with moAb214 and HRP-conjugated goat anti-mouse IgG antibody (DAKO). Upon addition of the peroxidase substrate *o*-phenylene-diamine (Kem-*En*-Tec Diagnostics), a colored reaction was developed; the reaction was stopped with 0.1 M H_2SO_4, and the optical density read at 492 nm. The concentration of plasma FH was estimated by comparison with reference plasma containing a known FH amount.

2.3. Genetic studies

Studies to identify mutations and risk variants were performed on the patients with aHUS. Each exon of the *CFH* (Pérez-Caballero et al., 2001), *MCP* (Richards et al., 2003), *CFI* (Fremeaux-Bacchi et al., 2004), *CFB* (Goicoechea de Jorge et al., 2007), *C3* (Martínez-Barricarte et al., 2012), *THBD* (Delvaeye et al., 2009) and *DGKE* (Sánchez Chinchilla et al., 2014) genes was amplified from genomic DNA using specific primers derived from the 5′ and 3′ intronic sequences as described. Polymerase chain reaction (PCR) fragments were sequenced to identify mutations and to determine the frequency of the *CFH*(*H3*) and *MCPggaac* aHUS-risk haplotypes. The *CFHR1*B* allele, conferring increasing susceptibility to aHUS, was identified by PCR generation and direct sequencing of *CFHR1* exon 6 (Abarrategui-Garrido et al., 2009). The presence of the common $\Delta_{CFHR3\text{-}CFHR1}$ allele, as well as copy number variations in the *CFH-CFHR1* to *CFHR5* genomic region were analyzed by Multiplex ligation-dependent probe amplification (MLPA), using the P236 A1 ARMD mix 1 from MRC-Holland (Amsterdam, Netherlands). Copy number variations of the *CFH* and *CFHRs* genes were also analyzed in some cases, using a recently developed, in-house CGH microarray (Tortajada et al., 2013).

CFHR3 exon 5 was generated from genomic DNA by using forward (5′-TTGAAAATGCAGATGTCTTCC-3′) and reverse (5′-GAACTCCTGACCTCATGG-3′) PCR primers and FideliTaq DNA polymerase (USB). PCR conditions were as follows: 94 °C for 5 min; 35 cycles (94 °C/30 s, 56 °C/15 s, 68 °C/10 s); 68 °C for 7 min. Excess primers and deoxyribonucleotides (dNTPs) were digested with Exonuclease I and shrimp alkaline phosphatase (USB). Direct