Complete functional characterization of disease-associated genetic variants in the complement factor H gene

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Genetic analyses in atypical hemolytic uremic syndrome (aHUS) and C3-glomerulopathy (C3G) patients have provided an excellent understanding of the genetic component of the disease and informed genotype-phenotype correlations supporting an individualized approach to patient management and treatment. In this context, a correct categorization of the disease-associated gene variants is critical to avoid detrimental consequences for patients and their relatives. Here we describe a comprehensive procedure to measure levels and functional activity of complement regulator factor H (FH) encoded by *CFH*, the commonest genetic factor associated with aHUS and C3G, and present the results of the analysis of 28 uncharacterized, disease-associated, FH variants. Sixteen variants were not expressed in plasma and eight had significantly reduced functional activities that impact on complement regulation. In total, 24 of 28 *CFH* variants were unambiguously categorized as pathogenic and the nature of the pathogenicity fully documented for each. The data also reinforce the genotype-phenotype correlations that associate specific FH functional alterations with either aHUS or C3G and illustrate important drawbacks of the prediction algorithms dealing with variants located in FH functional regions. We also report that the novel aHUS-associated M823T variant is functionally impaired. This was unexpected and uncovered the important contribution of regions outside the N-terminal and C-terminal functional domains to FH regulatory activities on surfaces. Thus, our work significantly advances knowledge towards a complete functional understanding of the *CFH* genetic variability and highlights the importance of functional analysis of the disease-associated *CFH* variants.

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Factor H (FH) is the key regulator of the alternative pathway (AP) of the complement system. FH controls complement activation, both in the fluid phase and on cellular surfaces, preserving complement homeostasis and preventing uncontrolled C3b deposition and host tissue damage. The FH regulatory activities include binding to C3b, blocking C3b-factor B interaction during the assembly of the AP C3-proconvertase complex (C3bB); acceleration of the decay of the AP C3-convertase (C3bBb); and acting as cofactor of factor I (FI) in the proteolytic inactivation of C3b.[1] FH is a relatively abundant plasma protein that is secreted as a single-chain glycoprotein of 155 kDa composed of 20 homologous domains of ~60 amino acids, called short consensus repeats (SCRs). FH concentration in plasma is highly variable, ranging from 76 to 247 μg/ml. Along the 20 SCR of FH, there are different interaction sites for C3b and polyanions. The SCR1-4 region is the only C3b binding site acting as a cofactor for FI to cleave C3b and to accelerate the decay of AP C3-convertase. Similarly, the C3b and polyanion binding site at SCR19-20 determines the ability of FH to bind surface-bound C3b, this region of FH being essential for self-pathogen discrimination.[2,3] In addition to these regions, it was recently postulated that a central FH segment, including SCR14, facilitates FH bending and increases avidity for C3b by enabling the simultaneous binding of FH to different sites in C3b.[4,5]

Pathogenic variants and polymorphisms in the *CFH* gene are associated with a number of diseases, including atypical hemolytic uremic syndrome (aHUS) and C3-glomerulopathy (C3G).[6] aHUS is a thrombotic microangiopathy characterized by thrombocytopenia, hemolytic anemia, and acute renal failure. The primary pathogenic event in aHUS is endothelial cell injury caused by complement dysregulation.[7] C3G is a very rare form of glomerulonephritis, characterized by the presence of electron-dense deposits within the glomerular basement membrane.[8] C3G is associated with complement abnormalities that lead to a persistent reduction of C3 serum levels and to intense deposition of degradation products of C3 in the glomerular basement membrane.[9,10]

Over the past 15 years, genetic analyses in aHUS and C3G patients have shown that variants in *CFH* are the most common genetic alteration associated with these disorders. However, we have a limited understanding of the functional

consequences of almost one-third of the genetic variants identified in *CFH*, which is a potential cause of misinterpretations with important consequences for the patients and their relatives. We report here the development of a comprehensive analytical procedure for the functional characterization of FH and demonstrate its effectiveness in identifying the expression and functional consequences of a large number of novel aHUS and C3G-associated *CFH* variants. The data allowed us to categorize them as pathogenic or benign and reinforced our current understanding of the pathogenic mechanisms underlying aHUS and C3G.

RESULTS

Novel FH variants found in the genetic analysis of aHUS and C3G patients

A total of 707 aHUS and 234 C3G patients included in the Spanish aHUS/C3G Registry (https://www.aHUSC3G.es) were screened for *CFH* variants, which resulted in the identification of 101 *CFH* variants, 87 in aHUS and 35 in C3G; 21 variants were associated with both pathologies. As a whole, 123 aHUS patients (17.4%) and 27 C3G patients (11.5%) carried *CFH* gene variants. Notably, 28 of these *CFH* variants (27.5%) were novel variants for which the functional consequences were unknown (Table 1). In addition, these patients were screened for all aHUS and C3G candidate genes. A summary of the clinical and genetic characteristics of the patients carrying these novel *CFH* variants is presented in Table 2 to illustrate that most patients presented with severe disease phenotypes. All other *CFH* variants ($N = 73$) found in our patients were previously described, and their functional consequences are known.[11] We named those variants located in SCR1-4 (R53C, R53P, R53S, S58A, C66Y, L98F, A110S, Y118Ifs*4, C192W, and Y235C) N-terminal, those in SCR19-20 (D1119N, P1166L, R1182K, C1218R, and c.3493+1G>A) were named C-terminal, and variants located in SCR5-18 (C309W, C320R, K331E, C597*, K670T, M823T, R830W, C853R, C853Y, R885Sfs*13, T956M, W1037*, and c.2957-1G>A) were named central region. A number of asymptomatic relatives carrying the *CFH* gene variants were also identified (Table 1). A search for these *CFH* variants in the Spanish and European control populations illustrated, that with only 3 exceptions (S58A, R830W, and T956M), all other *CFH* variants have not been previously encountered (Supplementary Table S1).

To predict the functional consequences of the 28 novel *CFH* variants detected in this study, we used several prediction algorithms available in ANNOVAR software. We considered likely pathogenic those variants in which a deleterious impact of the genetic variation was anticipated by a majority of these prediction algorithms. According to these analyses, the *CFH* variants R53C, R53S, C66Y, C192W, Y235C, C309W, C320R, C853R, C853Y, D1119N, and C1218R were considered likely pathogenic, whereas R53P, S58A, L98F, A110S, K331E, K670T, M823T, R830W, T956M, P1166L, and R1182K were predicted to be benign or variants of uncertain significance (Supplementary Table S2). For the C597*, W1037*, Y118Ifs*4, and R885Sfs*13 variants, some of the prediction algorithms failed to give a result. However, the nature of these *CFH* variants, introducing a stop codon, justifies their assignment to the category of likely pathogenic variants. Similarly, c.2957-1G>A and c.3493+1G>A were predicted to be pathogenic because they alter the splicing sequences.

As a whole, the analyses to predict pathogenicity indicate that 11 of the 28 *CFH* variations (39%) are predicted to be either benign or variants of uncertain significance (Supplementary Table S2). To formally categorize the disease-associated *CFH* variants and to establish their causal relationship with the pathology, we determined their expression levels in plasma and purified those that were expressed to perform a complete functional characterization. To do this, we benefited from having serum or plasma samples from all patients and relatives and the Y402H genotypes available for all individuals (Table 1).

FH plasma levels

Twenty-one of the 28 *CFH* variants are carried by Y402H heterozygote individuals (patients and/or relatives). In all of these cases, a direct measurement of the FH produced by the mutated *CFH* allele was obtained. These analyses allowed us to conclude that the C66Y, Y118Ifs*4, C192W, Y235C, C309W, C320R, C597*, C853R, C853Y, R885Sfs*13, W1037*, and C1218R *CFH* variants are not expressed or result in very low levels of FH in plasma. We also concluded that the L98F, c.2957-1G>A, and c.3493+1G>A variants are not expressed because total plasma levels in heterozygote carriers were <50% of the normal FH plasma levels (Supplementary Figure S1). In addition, we found that the A110S *CFH* variant, albeit present in plasma, it is significantly reduced compared with normal FH levels (Table 1 and Supplementary Figure S1). The normal range of variation for FH plasma levels produced by a single allele and the normal range for total FH plasma levels in control individuals were previously determined to be 50 to 127 μg/ml and 76 to 247 μg/ml, respectively (Supplementary Figure S1).

To functionally characterize the *CFH* variants that are expressed (R53C, R53S, S58A, K331E, K670T, T956M, and P1166L), we purified them to homogeneity from the plasma or serum of Y402H heterozygote carriers (Figure 1b). The expressed *CFH* variants for which no Y402H heterozygote carriers were available (R53P, M823T, R830W, D1119N, and R1182K) were purified together with the wild-type FH protein (Figure 1c).

Alternative pathway regulatory activity of the disease-associated FH variants

Six of the 12 purified FH variants (R53S, S58A, M823T, D1119N, P1166L, and R1182K) present significant reduced capacity to regulate the activity of the alternative pathway in our modified sheep red blood cell (SRBC) hemolytic assay (Figure 2). Notably, K331E showed significantly reduced lysis, which suggests improved surface protective capacity. It should also be mentioned that among the 5 variants that showed no