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Original Article

Functional analysis of rare genetic variants in complement factor I in advanced age-related macular degeneration

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Abstract

Factor I (FI) is a serine protease inhibitor of the complement system. Heterozygous rare genetic variants in complement factor I (*CFI*) are associated with advanced age-related macular degeneration (AMD). The clinical impact of these variants is unknown since a majority have not been functionally characterized and are classified as 'variants of uncertain significance' (VUS). This study assessed the functional significance of VUS in *CFI*. Our previous cross-sectional study using a serum-based assay demonstrated that *CFI* variants in advanced AMD can be categorized into three types. Type 1 variants cause a quantitative deficiency of FI. Type 2 variants demonstrate a qualitative deficiency. However, Type 3 variants consist of VUS that are less dysfunctional than Types 1 and 2 but are not as biologically active as wild type (WT). In this study, we employed site-directed mutagenesis followed by expression of the recombinant variant and a comprehensive set of functional assays to characterize nine Type 3 variants that were identified in 37 individuals. Our studies establish that the expression of the recombinant protein compared with WT is reduced for R202I, Q217H, S221Y and G263V. Further, G362A and N536K, albeit expressed normally, have significantly less cofactor activity. These results led to re-categorization of *CFI* variants R202I, Q217H, S221Y and G263V as Type 1 variants and to reclassification of N536K and G362A as Type 2. The variants K441R, Q462H and I492L showed no functional defect and remained as Type 3. This study highlights the utility of an in-depth biochemical analysis in defining the pathologic and clinical implications of complement variants underlying AMD.

Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness worldwide, and the number of people with AMD is projected to increase from 196 million in 2020 to 288 million in 2040 (1). This slowly progressive, neurodegenerative disease of the retina usually manifests after 60 years of age and can markedly reduce the quality of life. Loss of central vision can occur secondary to disruption and death of photoreceptor cells in the macula related to atrophy and/or neovascularization. While diet, smoking and other behavioral factors are associated with the risk of advanced AMD (2), it has become increasingly evident that polymorphisms and rare variants in genes encoding complement system proteins factor H (FH) and factor I (FI) as well as C3 and C9 play a predisposing role (3–9).

The complement system not only defends the host against microbes but also facilitates the processing and clearance of damaged/altered cells and tissue debris. Complement is activated via three major cascades: the classical, lectin and alternative pathways. Each is uniquely engaged but they share the common goal of promoting the inflammatory response and altering the membranes of tissue targets. Because the complement cascade provides a rapid response system as well as a potent surveillance and effector mechanism, strict control is required to avoid damage to self-tissue. Thus, inhibition of complement activation is mediated by both plasma and cell-bound regulators. For example, the serine protease FI (Fig. 1A), in conjunction with cofactor proteins such as FH or membrane cofactor protein (MCP, CD46), modulates the complement cascade through proteolytic cleavage of C3b generated by each pathway (Fig. 1B and C). The prevailing hypothesis is that an overly exuberant inflammatory response driven by an inadequately regulated complement cascade resulting primarily from genetic alterations is a major player in the pathophysiology of AMD (3–11). Thus, for a given degree of injury, there is an excessive degree of complement activation which is damaging to the retina.

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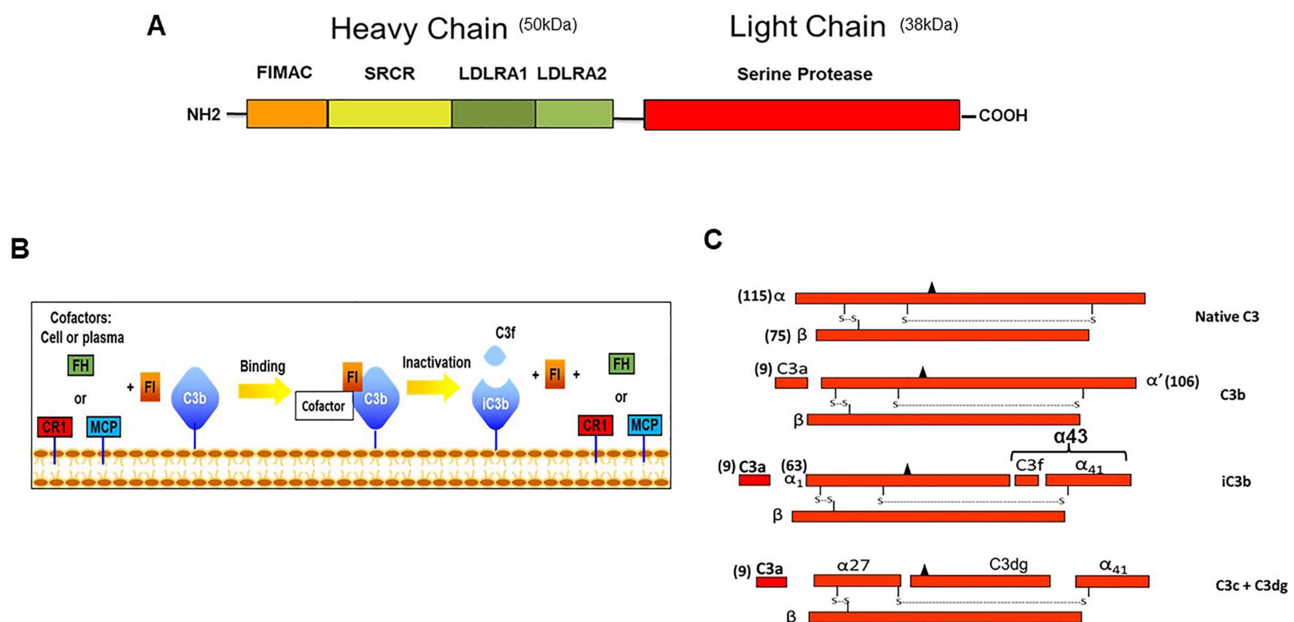

Figure 1. (**A**) Schematic diagram of FI domain organization. FIMAC, FI membrane attack complex; SRCR, scavenger receptor cysteine-rich; LDLRA1, low-density lipoprotein class 1; LDLRA2, low-density lipoprotein class 2. (**B** and **C**) C3b binding and cofactor activity. FI, in the presence of a cofactor protein (FH, MCP; CD46), or CR1 (CD35), cleaves the α' chain of C3b to obtain α43 and α41 (liberating C3f, a 2-kDa fragment), forming iC3b. Next, CR1 achieves further cleavage of iC3b to C3c and C3dg.

This undesirable tissue response to injury may affect the retinal pigment epithelium, drusen formation, and/or the local vasculature leading to a predisposition to and an acceleration of the disease process.

Genetic variants in complement factor I (*CFI*) and *CFH* associated with AMD range from common polymorphisms, conferring relatively low to moderate risk, to rare variants with nearly complete penetrance and high risk. Also, the rare variants with high impact lead to younger age of onset of AMD (12–15). The rare *CFH* variant R1210C is associated with a high burden of drusen in the macular and extramacular regions (13) and this variant and the C3 rare variant (K155Q) are associated with an enhanced rate of progression from early and intermediate stages to advanced stages of AMD as well as earlier age of progression (14,15). The association between AMD and a common variant in *CFI* was first implicated in 2009 by Fagerness *et al.* (16). In 2013, a targeted sequencing study of 681 genes and 2493 individuals led to the discovery of a burden of 59 rare *CFI* variants associated with AMD, and loss of function variants had the greatest impact on increasing risk of AMD (4). Studies have also noted a strong predisposition to advanced AMD in those with reduced serum FI levels, and, in a few cases, a reduction in catalytic activity has been implicated (3–5). However, a majority of the variants in the *CFI* gene were incompletely characterized.

We have recently published functional studies of *CFI* variants using a serum-based assay that facilitated establishing the clinical relevance of the variants and further stratified them according to the assay into Type 1, 2 or 3 *CFI* mutations (17). Type 1 variants led to low serum FI antigenic levels (FI protein is not made in normal amounts) and a corresponding decrease in FI function. Type 2 variants demonstrated normal serum FI antigenic levels but reduced functional activity (FI protein is made in normal amounts but doesn't function). Thus, while variants belonging to Types 1 and 2 were clearly deleterious, Type 3 variants demonstrated a functional deficiency that was less defective than the Type 2 variants but was not equivalent in regulatory activity to wild type (WT). In this study, we employed site-directed mutagenesis followed by expression of the recombinant variant and then an assessment of the functional repertoire to clarify the significance of Type 3 variants in order to better predict the risk of AMD development in these individuals.

Results

Our previous studies using serum-based assays categorized *CFI* variants in individuals with and without advanced AMD into three groups based on antigenic and functional assessments (17). The function of FI was determined by measuring the proteolytic cleavage of C3b to iC3b, utilizing the cofactor protein, FH (17). Type 1 variants demonstrated low serum FI levels and a corresponding decrease in C3b degradation. Type 2 variants showed normal serum FI antigenic levels yet a striking reduction (∼80%) in the degradation of C3b to iC3b. Type 3 variants had normal antigenic levels and a more modest reduction in cofactor activity as iC3b generation was low only when measured per unit of FI. A majority of these Type 3 variants were classified as 'variants of uncertain significance (VUS)' based on the American College of Medical Genetics

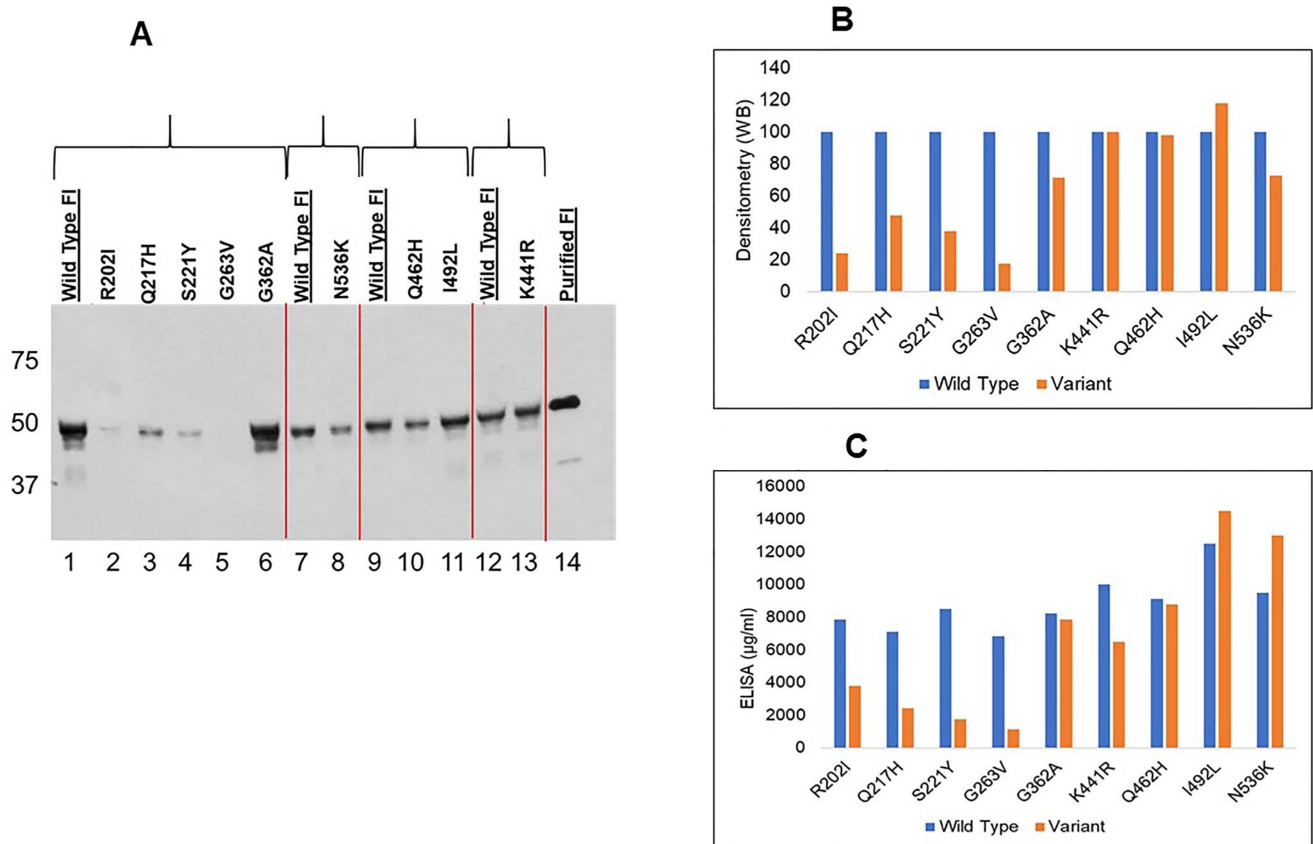

Figure 2. Biosynthetic evaluation of transfected variants. (**A**) Western Blotting of reduced SDS-PAGE of supernatants from transfected WT and variants are shown. For each expression study, indicated by the brackets and separated by red lines, both the variant(s) and WT FI were conducted. Controls for the Western Blotting also utilized purified FI (lane 14) that demonstrates the heavy chain at 50 kDa and the light chain at 38 kDa. Similar banding pattern is seen for the WT FI expression controls (lanes 1, 7 and 9). Recombinant expression of *CFI* genetic variants R202I (lane 2), Q217H (lane 3), S221Y (lane 4) and G263V (lane 5) shows decreased secretion compared with the WT control (lane 1). The secretion of the remaining variants is comparable to their WT [G362A (lane 7) and N536K (lane 8) to WT (lanes 1 and 9), respectively; Q462H (lane 10) and I492L (lane 11) to WT (lane 9); K441R (lane 13) to WT (lane 12)]. R, arginine; I, isoleucine; Q, glutamine; H, histidine; S, serine; Y, tyrosine; G, glycine; V, valine; A, alanine; N, asparagine; K, lysine; L, leucine. (**B**) Bar charts demonstrating comparison of WT and variant secretion based on Western Blotting. (**C**) Bar charts demonstrating comparison of WT and variant secretion based on densitometry and on ELISA. Also, see Table 1.

(ACMG) guidelines (18). Therefore, to further define their clinical significance, the nine uncharacterized Type 3 variants (R202I, Q217H, S221Y, G263V, G362A, K441R, Q462H, I492L and N536K) identified in 37 individuals were recombinantly produced and, if expressed, their function was characterized relative to the WT protein.

CFI variants R202I, Q217H, S221Y and G263V

As assessed by enzyme-linked immunosorbent assay (ELISA), the secretion of the recombinant protein by 293 T cells compared with WT was reduced for R202I by 52%, Q217H by 76%, S221Y by 80% and G263V by 84% (Table 1). This expression level obtained by ELISA was further confirmed by Western Blotting (WB) (Fig. 2A–C). Eleven individuals carried the R202I variant (four with advanced AMD and seven without AMD) and one of the four with AMD had a low normal FI antigenic level (35.1 μg/ml; normal range 29.3–58.5 μg/ml). Of the seven without AMD, two individuals had a family history of AMD. There was one individual each with Q217H, S221Y or G263V and they expressed normal FI antigenic levels in serum (Q217H, 50.6 μg/ml; S221Y, 33.1 μg/ml; G263V, 55.3 μg/ml; normal range 29.3–58.5 μg/ml). Of these, the individual carrying Q217H in our cohort did not have AMD and had no family history of AMD. Although these results raised the question whether decreased secretion of FI *in vitro* in 293 T cells accurately translates to low serum antigenic levels in patients, we note that these three variants have been reported previously in AMD patients with low antigenic levels (19). Therefore, we speculate that the normal serum level in our patients is likely reflective of an increase in the secretion of the WT allele (indicative of the acute phase nature of FI) and thereby 'masked' a Type 1 variant.

Structural analysis further verified that R202 did not directly interact with FH (Fig. 3A–C); however, the side chain of residue R202 is exposed to the solvent and makes a salt-bridge with residue D303, which is part of the activation domain of FI. Given that R to I substitution replaces a charged amino acid with a hydrophobic one, we expect a significant local perturbation of the structure and, perhaps, lower protein expression by reducing solubility of the polypeptide chain. Structural analyses for residues Q217 and S221 demonstrated that they are

Table 1. Expression of recombinant WT and variant proteins as determined by ELISA and Western Blotting

Variant	ELISA (μg/ml)—mean ± SEM (95% confidence interval)	Densitometry—[quantification of FI band on WB (%)]	Secretion—compared with WT (%)
WT	7875 ± 2418	100	Decreased > 50
R202I	3800 ± 1529	24.0	
WT	7125 ± 2085	100	Decreased > 50
Q217H	2425 ± 337	47.8	
WT	8500 ± 4500	100	Decreased > 50
S221Y	1750 ± 1750	38.2	
WT	6833 ± 3086	100	Decreased > 50
G263V	1133 ± 592	17.6	
WT	8250 ± 4750	100	Comparable
G362A	7871 ± 3129	71.5	
WT	10 000 ± 6000	100	Comparable
K441R	6500 ± 3500	100	
WT	9133 ± 2652	100	Comparable
Q462H	8783 ± 5766	98	
WT	12 500 ± 2500	100	Comparable
I492L	14 500 ± 7500	118	
WT	9500 ± 5500	100	Comparable
N536K	13 000 ± 8000	72.8	

in the linker connecting the SRCR (scavenger receptor cysteine-rich) and LDLR1 (low density lipoprotein receptor) domains of the heavy chain of FI (Fig. 3A–C). In the structure of the free form of FI (20), electron density is weak for Q217 and missing for S221, suggesting flexibility of this region, whereas they are visible in the structure of FI in complex with FH and C3b, implying rigidification upon binding. Mutations of residues Q217 and S221 could therefore introduce structural constraints to the linker resulting in impaired folding and thereby decreased secretion. Structural analysis for residue G263 indicated that it lies in the LDLR2 domain, sandwiched between S274 and the disulfide bond C259-C271. Given the tight space, G is the only amino acid that can fit without disrupting this three-dimensional organization. Mutation of G to a bulkier amino acid such as V is expected to negatively affect formation of the disulfide bond, which is important for protein folding and stability.

Our structure–function data thus ascertained that the variants R202I, Q217H, S221Y and G263V are likely deleterious owing to a quantitative deficiency of FI (although the patients in our cohort had normal antigenic FI levels, likely owing to increased expression of the WT allele) (Table 2). These variants were therefore re-categorized as Type 1 (Table 3). Furthermore, based on our studies, Q217H, S221Y and G263V were also reclassified from VUS to 'likely pathogenic' and R202I as 'likely pathogenic with variable penetrance' per ACMG guidelines (Table 2).

CFI variants G362A and N536K

The secretion of G362A was normal compared with WT (Table 1, Fig. 2A–C). The one individual carrying this variant was 66 years old and had a low normal serum FI antigenic level (37.4 μg/ml; normal range 29.3–58.5 μg/ml). Although he had a family history of AMD, he did not have the disease. This variant has been previously reported in two patients with AMD (5,19,21) and shown to have low antigenic levels (21 μg/ml and 23.5 μg/ml; low plasma FI cutoff point of 23.69 μg/ml). The low antigenic levels in the setting of normal recombinant production may be reflective of increased turnover of this variant FI protein in blood, although this would need to be confirmed in future studies. Our recombinant data demonstrated that G362A had significantly decreased functional activity with MCP and CR1 but was borderline with FH (Fig. 4).

Similar to G362A, the variant N536K exhibited normal *in vitro* secretion compared with WT (Table 1, Fig. 2A–C). The one individual carrying the variant was 73 years old and did not have AMD but did have a family history of AMD, and had a normal serum FI antigenic level (42.9 μg/ml). This variant has also been reported previously in an AMD patient (19). Our recombinant data indicated that the functional activity of N536K was normal with FH and CR1 but defective when MCP was used as the cofactor protein (Fig. 5A and B).

Structural analysis reveals that G362 is part of a short loop (residues D359-T364) which favors binding of the substrate to the active site (Fig. 3A–C). Mutation of G to A, a bulkier amino acid, is likely to change the dynamics of this loop thereby restricting access to the substrate. Structural analysis for N536K points out that residue N536 does not participate in the cofactor/enzyme binding interface. However, the mutation removes an N-glycosylation site which likely accounts for the defective cofactor activity (22).

Our results thus established that G362A and N536K are likely deleterious based on defective function (Table 2). Both variants were therefore re-categorized as Type 2 (Table 3) and as 'likely pathogenic' based on the ACMG guidelines.

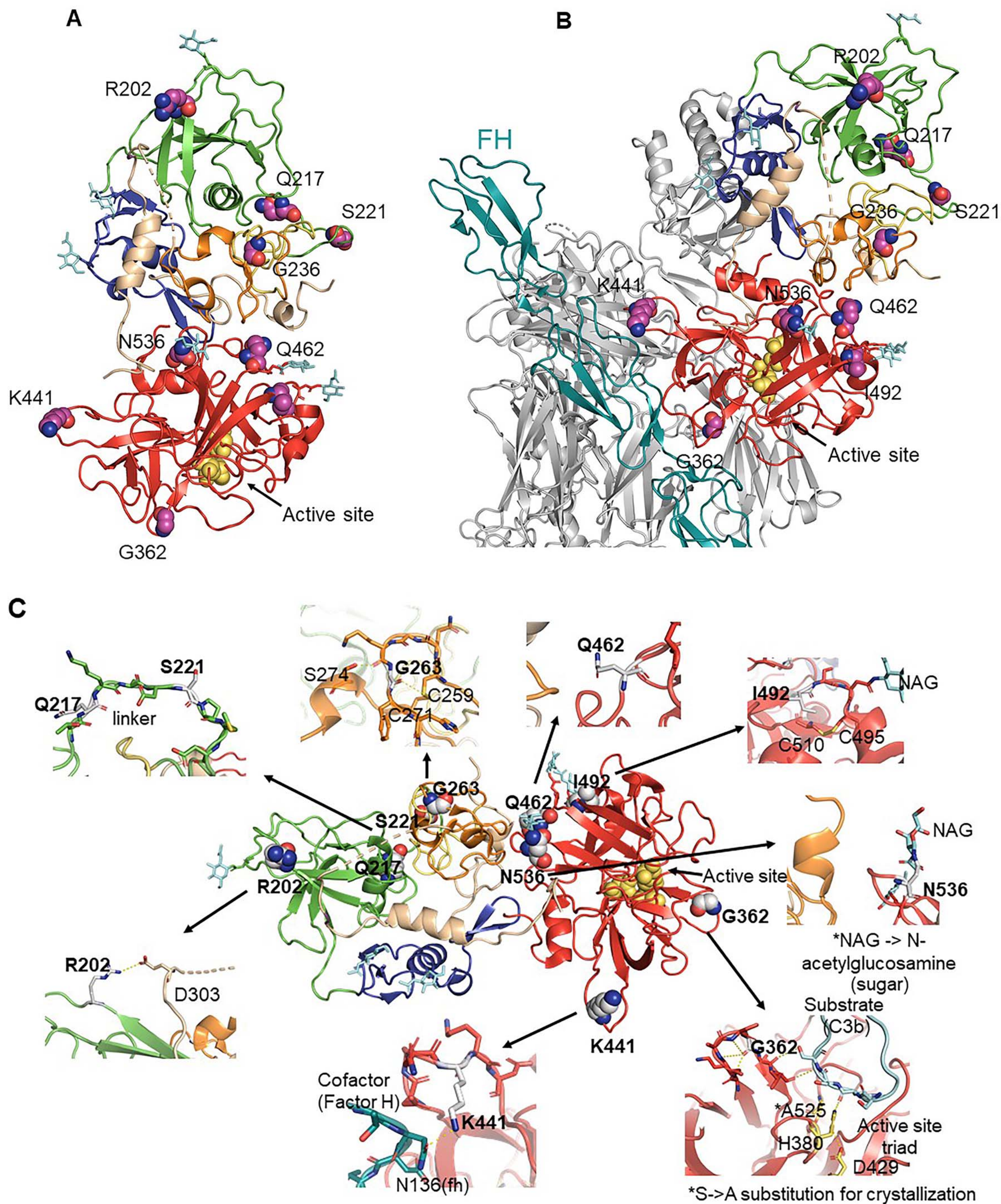

Figure 3. (**A** and **B**) Crystal structure of FI (generated using Pymol). Symbols indicate the following: purple circles = variant sites; yellow circles = serine protease site; light blue hexagons = N-glycosylation sites. Thick arrow-shaped structures in the various domains are beta pleated sheets. The dotted beige line in the linker represents a structure that is not visible in the crystal structure, and its location is therefore hypothetical. Serine protease, red; FIMAC, blue; linker, beige; LDLR1, yellow; LDLR2, orange; SRCR, green. **C.** Mapping of the FI variants on the surface of C3b in a complex with FI and FH (complement control repeats 1–4 and 19–20). Color coding is as follows: C3b, gray; FH, cyan. Refer to text for further explanation of the structural evaluation for each variant. R, arginine; Q, glutamine; S, serine; G, glycine; N, asparagine; K, lysine.

CFI variants K441R, Q462H and I492L

These Type 3 variants exhibited normal secretion compared with WT (Table 1, Fig. 2A–C) and no defect in function with any of the cofactors (Fig. 6). There were 19 individuals in our cohort who carried K441R (12 with AMD and seven without AMD). Two of 12 patients with AMD carrying K441R had a low serum FI antigenic level (23.6 μg/ml and 27 μg/ml; normal range, 29.3–58.5 μg/ml). Of the seven without AMD, three had a family history of AMD. The patient carrying I492L had AMD and a family history of AMD while the individual with Q462H did not have AMD and did not have a family history of

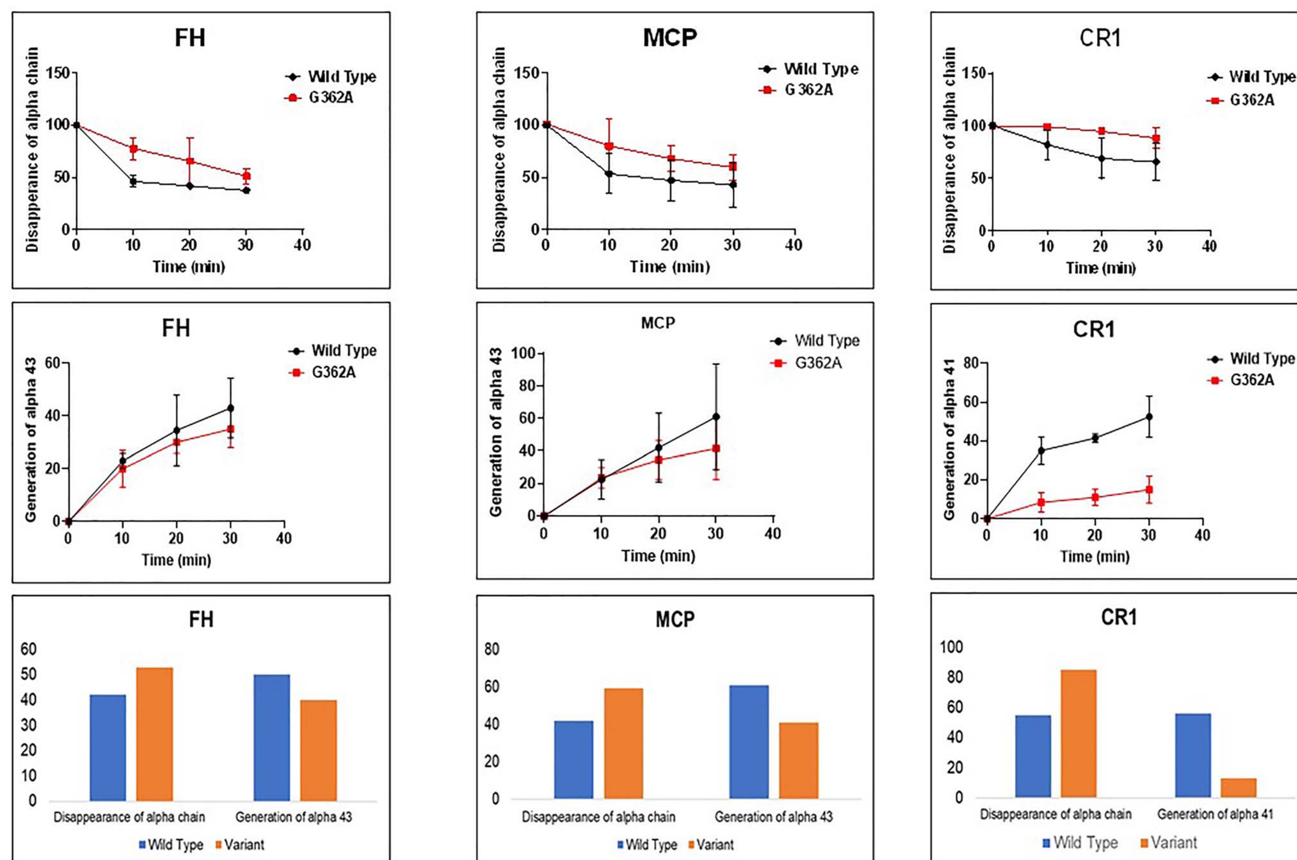

Figure 4. Functional evaluation of rare FI variant G362A: cofactor activity. The fluid-phase C3b cofactor activity of the variant FI with a cofactor protein (FH, MCP or CR1) was assessed by cleavage of purified C3b to iC3b and compared with WT FI derived from transfected cells. The percentage of alpha chain remaining and the percentage of generation of alpha 41 or alpha 43 indicates cleavage of C3b to iC3b at 0, 10, 20 and 30 min. Cleavage was quantitated by densitometric analysis of the remaining alpha chain and generation of alpha 43 or alpha 41. Data represent three separate experiments with bars corresponding to the SEM. Upon comparison to WT FI, the cofactor activity of variant G362A was significantly different with MCP and CR1 ($P < 0.05$) but not significant with FH as a cofactor (P 0.05). G, glycine; A, alanine. Bar charts demonstrating comparison of WT and variant cofactor activity at the 30 min time point. The numbers on the Y-axis in the bar charts reflect %.

Table 2. Summary of genetic and functional analyses of rare Type 3 *CFI* variants

Variant	Recombinant secretion (μg/ml)	Cofactor activity	ACMG interpretation	Modified ACMG interpretation (based on functional and structural analyses)
R202I	Decreased	Normal	VUS	Likely pathogenic with variable penetrance[b]
Q217H	Decreased	Not done[a]	VUS	Likely pathogenic
S221Y	Decreased	Not done[a]	VUS	Likely pathogenic
G263V	Decreased	Not done[a]	VUS	Likely pathogenic
G362A	Comparable to WT	Defective with MCP and CR1	VUS	Likely pathogenic
K441R	Comparable to WT	Normal	VUS	VUS[c]
Q462H	Comparable to WT	Normal	VUS	VUS
I492L	Comparable to WT	Normal	VUS	VUS
N536K	Comparable to WT	Defective with MCP	VUS	Likely pathogenic

[a]Since the secretion of Q217H, S221Y and G263V was reduced compared with WT (establishing a quantitative defect), cofactor activity was not conducted owing to the lack of secreted protein. [b]Four of 11 individuals carrying this variant had AMD. [c]Twelve of 19 individuals carrying this variant had AMD.

AMD. Both individuals had normal to low normal serum FI antigenic levels (I492L, 48.3 μg/ml; Q462H, 32.5 μg/ml; normal range, 29.3–58.5 μg/ml). These variants have been reported previously in AMD patients (5).

Structural analysis showed that the residue K441 is important for FH binding (Fig. 3A–C). However, K to R is a conservative mutation and less likely to adversely affect cofactor activity. Structural analysis demonstrated that residue I492 is next to the conserved disulfide bond C495–C510 (residues C168 and C182, chymotrypsinogen nomenclature), which, based on previous literature on the related serine protease thrombin (23,24), is likely

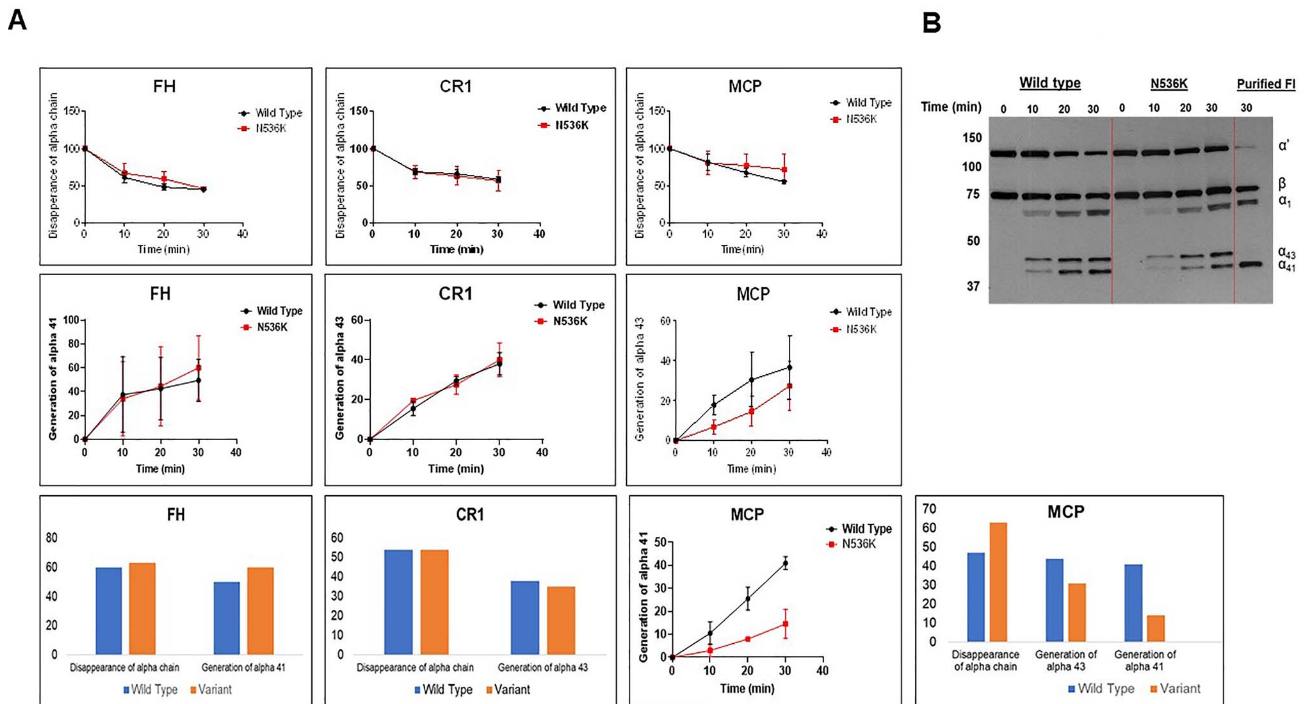

Figure 5. Functional evaluation of rare FI variant N536K: cofactor activity. (**A**) The fluid-phase C3b cofactor activity of the variant FI with a cofactor protein (FH, MCP or CR1) was assessed by cleavage of purified C3b to iC3b and compared with WT FI derived from transfected cells. Data represent three separate experiments with bars corresponding to the SEM. There was no significant difference in the percentage of alpha chain remaining between WT and N536K variant for FH ($P > 0.05$) and CR1 ($P > 0.05$). The *P*-value for the difference in the percentage of alpha chain remaining between WT and N536K variant for MCP was not significant ($P = 0.07$) but reached significance for alpha 43 generation ($P < 0.05$). Bar charts demonstrate a comparison of WT and variant cofactor activity at the 30 min time point. The numbers on Y-axis reflect %. (**B**) Representative Western Blotting for MCP.

Table 3. Reclassification of rare Type 3 *CFI* variants

Variant	Re-categorization of Type 3 variants based on current functional and structural analysis
R202I	Type 1
Q217H	Type 1
S221Y	Type 1
G263V	Type 1
G362A	Type 2
K441R	Type 3
Q462H	Type 3
I492L	Type 3
N536K	Type 2

Type 1 variants cause a quantitative deficiency of FI (haploinsufficiency secondary to a decreased FI antigenic level with a proportional decrease in function). Type 2 variants demonstrate a qualitative deficiency (normal FI antigenic level but decreased function). Type 3 variants consist of 'VUS' that are less dysfunctional than Types 1 and 2 but are not as biologically active as WT. See text for detailed explanation of the structural and functional assessments that led to the reclassification of these variants.

important for the stability and function of the enzyme. However, since I to L is a conservative mutation, this substitution may be tolerated by the enzyme. Residue Q462 is at the interface between the light and heavy chain of FI. Since it is unclear how the heavy chain influences the catalytic activity of the light chain, no definitive conclusions can be derived from the structural data. Overall, our work substantiated that these variants were not deleterious. However, owing to rarity of the variants they remained VUS per ACMG guidelines and classified as Type 3 (Tables 2 and 3).

Discussion

Rare genetic variants in *CFI* have been associated with advanced AMD (4–6,11). However, a majority of the variants have not been functionally characterized and many are classified based on *in silico* analyses as VUS or likely benign. This inability to ascertain the function of genetic variants impairs our understanding of disease pathogenesis and is an impediment to personalized management of AMD.

In our prior work, we implemented a high throughput serum-based functional assay to determine the significance of FI variants that led to their classification into three main types (17). Variants belonging to Types 1 and 2 were deleterious (not synthesized or synthesized but had marked loss of function); however, the functional significance of Type 3 variants was not entirely clear since they demonstrated a modest decrease in function with normal antigenic levels. Several individuals carrying Type 3 variants also did not have AMD, although it was present in their family members.

Therefore, to more rigorously assess their functional repertoire, and to better predict the risk of AMD development in these individuals, we produced the nine Type 3 variants recombinantly and analyzed their function utilizing multiple cofactor proteins. This systematic analysis led to the re-categorization of four variants (R202I, Q217H, S221Y and G263V) as Type 1 and two (G362A and N536K) as Type 2. Of the remaining variants, K441R,

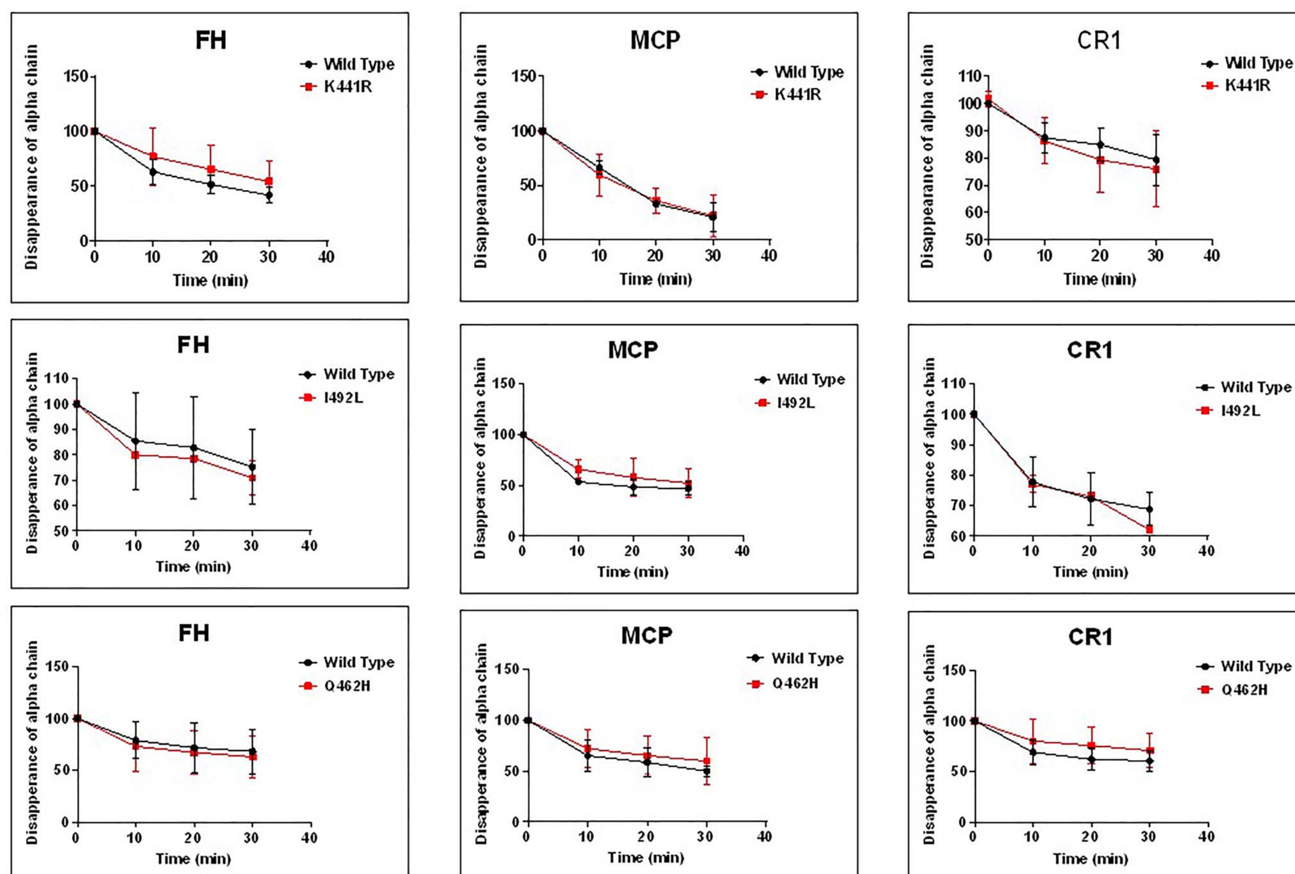

Figure 6. Functional evaluation of rare FI variants K441R, I492L and Q462H: cofactor activity. The fluid-phase C3b cofactor activity of the FI variants K441R, Q462H and I492L with a cofactor protein (FH, MCP or CR1) was assessed by cleavage of purified C3b to iC3b and compared with WT FI derived from transfected cells. Data represent three separate experiments with bars corresponding to the SEM. There was no significant difference ($P > 0.05$) in the percentage of alpha chain remaining between WT and variants for either of the cofactors, FH, MCP or CR1.

Q462H and I492L demonstrated no defect in secretion or function but the ACMG classification remained a VUS owing to the rarity of the variants. These variants were thus left classified as Type 3.

These results highlight a potential problem in using the ACMG guidelines to interpret variants. These guidelines are designed for Mendelian diseases and heavily weigh population frequency. Based on these results, we suggest that modifications to the ACMG framework for diseases with variable penetrance, such as AMD, be refined and extended. One such initiative is the National Institutes of Health (NIH)-funded Clinical Genome Resource (ClinGen). A specific goal of ClinGen is to develop teams of experts to evaluate the clinical validity of gene-disease relationships and pathogenicity of individual genetic variants. These clinical domain working groups (CDWG) will implement standardized protocols for gene or sequence variant specific annotations of genes related to the specific disease domain. The guidelines from these groups, CDWG and ACMG, in conjunction with detailed structure–function analyses (such as described here), will eventually help to clarify the significance of genetic variants in various diseases (18).

As an example, about 40% of the rare genetic variants in our advanced AMD cohort have been observed in patients with atypical hemolytic uremic syndrome (aHUS), a thrombotic microangiopathy in which rare mutations in *CFI* are causative in 5–15% of patients (18,25). This association of variants causing two apparently disparate diseases is striking. Atypical hemolytic uremic syndrome features acute endothelial injury that often arises in early childhood and young adults, whereas AMD features biological debris in the retinal pigment epithelium, and is a disease manifested primarily in older adults. We did not find a pattern of association with kidney disease in the individuals carrying the variants evaluated in this study. However, we have reported an association between reduced kidney function and advanced AMD in a study of the NHANES population (26). Gaining insights into the functional capabilities of these variants may help to delineate patients who are at risk of developing acute kidney disease early in life versus those who are at risk of manifesting another chronic debilitating condition at an older age.

In summary, our results establish that the strategy of recombinant protein production followed by systematic functional and structural assessment provides substantial insights into the clinicopathologic significance of genetic variants and their role in pathophysiology of AMD as well as other complement-mediated diseases. Such a model could provide critical guidance relative to an individualized treatment plan for these patients.

Identification of individuals with advanced AMD at a younger age, who have dysfunctional *CFI* variants, will facilitate screening and targeting those most likely to benefit from complement pathway related therapies.

Materials and Methods

Patients

Individuals with and without rare (minor allele frequency < 1%) heterozygous *CFI* genetic variants were identified based on our previous studies (4–6). The number of individuals carrying a rare *CFI* variant was 231 in a cohort of 3666 subjects (6.3%). Of the 231, serum samples were available for 106 individuals (78 with advanced AMD and 28 without advanced AMD). The comparison cohort included 93 non-carriers of rare variants in *CFI* (49 with advanced AMD and 44 without advanced AMD) matched to carrier individuals by age at date of sample collection within two years. We utilized a novel serum-based assay to characterize the function of the FI variants in the 106 individuals (17). Eighteen variants identified in 35 patients with advanced AMD belonged to Type 1, six variants in seven individuals (six with advanced AMD and one without AMD) belonged to Type 2 and 15 variants in 64 individuals (37 with advanced AMD and 27 without advanced AMD) belonged to Type 3 (17). Functional analyses for six Type 3 variants have been previously reported (27). In this study, we characterized the other nine Type 3 variants (R202I, Q217H, S221Y, G263V, G362A, K441R, Q462H, I492L and N536K) using the assays described below.

Variant classification

The variants were also classified based on the guidelines established by the joint consensus of the ACMG and the Association of Molecular Pathology (AMP) (18). Based on these standards, variants are classified as pathogenic; likely pathogenic; VUS; likely benign and benign. NM_000204.5 was used as the reference transcript for *CFI*. Recommendations from the ClinGen Low Penetrance/Risk Allele Working Group (https://clinicalgenome.org/site/assets/files/4531/clingenrisk_terminology_recomendations-final-02_18_20.pdf) were also considered. Variant classification was performed by a board-certified clinical laboratory geneticist.

Mutagenesis

A 6x histidine carboxyl-terminal tagged *CFI* cDNA cloned in pcDNA3 was purchased from GenScript (Piscataway, NJ, USA). The variants were produced using the QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Stratagene, Santa Clara, CA, USA). All cDNA clones were sequenced in their entirety to assure fidelity.

Expression of recombinant FI mutants

Human embryonic kidney 293 T cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA, USA) containing 10% heat-inactivated fetal calf serum (FCS, Harlan, Madison, WI, USA), L-glutamine (2 mM, Sigma, St. Louis, MO, USA), penicillin and streptomycin (10,000 U/ml and 100 mg/ml, Cellgro® Mediatech), non-essential amino acids and sodium pyruvate. Short-tandem repeat (STR) profiling of human cell lines has been used to authenticate or confirm the identity of these cells. The STR analysis at ATCC meets requirements for funding, publication and quality control. For transfection, the DMEM was replaced with OptiMEM® (Invitrogen, NY, USA). Transient transfections were performed using X-tremeGENE HP DNA transfection reagent (Roche) and supernatants were collected after 48 h. The recombinant FI produced from mammalian cells was variably secreted as both a single polypeptide chain, i.e. pro-I(s), and as a mature FI composed of a disulfide-linked heavy chain and light chain. To overcome this issue and also increase the yield of mature FI, we co-transfected FI with furin cDNA cloned in pCMV (25,28). Quantitation and characterization of FI in the eluate were performed by sandwich ELISA and Western Blotting. Transfections were performed at least three times for each variant.

Cell lysates

Cells (4×10^7 ml) were lysed in 1% NP-40, 0.05% SDS in TBS with 2 mM phenylmethane sulfonyl fluoride (PMSF) at 4°C for 30 min followed by centrifugation at 12 000 × g for 10 min. Supernatants were collected and stored at −80°C.

Quantification and Western Blotting (WB)

The quantity of each recombinant FI mutant protein was determined by ELISA. The capture antibody, OX21 (Serotec, Oxford, UK), was coated at 1 μg/ml overnight at 4°C and then blocked for 1 h at 37°C (1% BSA and 0.1% Tween 20 in PBS). Dilutions of WT, variant FI samples and purified human FI (Complement Technologies, San Diego, CA, USA) were incubated for 1 h and then washed with PBS containing 0.05% Tween- 20. Next, sheep anti-FI (Abcam, Cambridge, MA, USA) was applied for 1 h at 37°C. After washing, HRP-coupled rabbit anti-sheep IgG (Abcam, Cambridge, MA, USA) was added and incubated for 1 h at 37°C. After washing, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate (Pierce, Rockford, IL, USA) was added. Subsequently, the reaction was stopped using 2N H_2SO_4 and absorbance at 450 nm assessed.

WBs of supernatants and purified proteins were electrophoresed under reducing and non-reducing conditions using 10% SDS-PAGE, transferred to nitrocellulose and then probed with 1:5000 goat anti-FI Ab (Quidel, San Diego, CA, USA) followed by a 1:10 000 HRP-conjugated rabbit anti-goat IgG (Abcam, Cambridge, MA, USA). The polyclonal Ab to FI predominantly recognized epitopes on the heavy chain. Data were analyzed by using positive (purified proteins) and negative controls via WB and

ELISA. The WBs and ELISAs were conducted at least three times for each variant.

C3b cofactor assays

Cofactor assays were performed to assess the cleavage of human C3b by WT and FI variants Three major cofactors [FH, MCP, complement receptor 1 (CR1)] were used in the assays. For these assays, WT and FI variant proteins were diluted in physiologic salt (150 mM NaCl) or low salt (25 mM NaCl) buffer with C3b (10 ng; Complement Technologies, Inc, Tyler, TX, USA) at 37°C. Concentrations of WT or variant FI used with the individual cofactors were 10 ng with MCP, 20 ng with FH and 15 ng with CR1. Concentrations of cofactor used in the reactions were 100 ng MCP, 200 ng FH or 150 ng CR1. Reactions were carried out in a total volume of 15 μl/reaction at 37°C. Kinetic analysis of the WT and variants was achieved through collection of sample at 0, 5, 10 and 20 min. At each time point 7 μl of 3× Laemmli reducing sample buffer was added to individual reactions to stop the reaction and then heated at 95°C for 5 min. The samples were electrophoresed on 10% Tris-glycine gel and then transferred to nitrocellulose for WB analysis. Membranes were rinsed with TBS-T (0.05%Tween-20) for 5 min and blocked overnight with 5% non-fat dry milk in PBS. Blots were probed with a 1:5000 dilution of goat anti-human C3 (Complement Technologies, Inc, Tyler, TX, USA) followed by HRP-conjugated rabbit anti-goat IgG and developed with SuperSignal substrate (Thermo Fisher Scientific, Waltham, MA, USA). The signal detected on radiographic films was scanned using a laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). Multiple exposures were used to establish linearity. All assays were performed at least three times on each sample.

Structural analyses

The FI variants were mapped on the crystal structure of FI (S525A) bound to C3b and mini-FH (PDB ID: 5O32) (29) using the Pymol software version 2.4.0 (Schrodinger, LLC) (Fig. 3A–C).

Statistical analysis

To determine if a variant had a lower level of cofactor activity than WT, area under the curve (AUC) was computed for degradation of the α' chain of C3b and for the generation of the fragments α1, α41 or α43 between 0 min and 20 min. *P*-value was computed based on one-sided t-test for the difference of AUC between WT and variant FI. R programming language (CRAN, https://cran.r-project.org/) was used for statistical computations.

Commercial relationships disclosures

AJ is a consultant for Alexion Pharmaceuticals, Gemini Therapeutics, Novartis Pharmaceuticals and Chinook Therapeutics. She is also a principal investigator for the Apellis Pharmaceuticals.

JPA has Stocks, Equity or Options in Compliment Corporation, Kypha Inc, Gemini Therapeutics Inc and Q32 Bio and is also on the Scientific Advisory Board for these companies. JPA is a consultant for Celldex Therapeutics, Achillion Pharmaceuticals Inc, Annexon Biosciences Inc, Alexion Pharmaceuticals, BioMarin Pharmaceutical Inc, HiBIO Inc, Autobahn Therapeutic Inc, Takeda Pharmaceuticals & Janssen Research & Development LLC. He also serves on the Advisory Board for Broadwing Bio LLC and Arrowhead Pharmaceuticals Inc.

JMS is scientific co-founder of Gemini Therapeutics, Inc., has stock in Gemini Therapeutics and Apellis Pharmaceuticals, and is a consultant for Laboratories' THEA.

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Conflict of Interest statement. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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