Assessing the Functional Consequence of a *CFB* Variant in aHUS

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Introduction

- Atypical hemolytic uremic syndrome (aHUS) is a rare disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute kidney injury.
- Its pathogenesis is driven by dysregulated cell surface control of the alternative pathway (AP) of the complement cascade secondary to inherited and/or acquired factors.
- The complement cascade is tightly regulated by a host of complement regulators which maintain a low level of steady activation.
- Factor B (fB) is essential to AP activation. It interacts with C3b to form C3bB, which is cleaved by factor D (fD) to form the active but unstable C3 convertase (C3bBb).
- Variants in complement activators may confer resistance to regulation and/or increase C3 convertase activity.
- In this case study, two probands display a pattern of alternative pathway dysregulation and a novel heterozygous variant in *CFB* (c.1101 C>A, p.Ser367Arg).

Methods

- Complement biomarkers were measured by ELISAs and radial immunodiffusions. Classical and alternative pathway activities were assessed using Quidel and Wieslab kits, respectively. IFE was performed using Helena Titan Gel kit.
- Genetic testing was performed using targeted next-generation sequencing and Sanger sequencing.
- C3 deposition assay utilizes plated MES-13 cells in gelatin veronal buffer supplemented with Mg^{++} (0.5mM). C3 deposits were visualized using 7C12 antibodies which target C3.
- Proteins were produced using bacterial expression systems.
- Sheep Erythrocyte Decay Assays were performed using sheep erythrocytes, rat serum, and purified complement proteins.

Patients

- Patient one is a five year old Caucasian female who presented with schistocytes, thrombocytopenia, and low C3 at ten months old. Genetic testing identified a FHR1-FHR4 heterozygous deletion and the novel CFB variant, which also segregated in two second cousins with aHUS (**Fig. 1A**).
- Patient two is a fifty-five year old Caucasian female who presented with aHUS at thirty-one years old immediately following pregnancy (**Fig. 1B**). Genetic testing identified a heterozygous FHR3-FHR1 deletion, a variant in CFH (p.Gln950His), and the novel CFB variant.

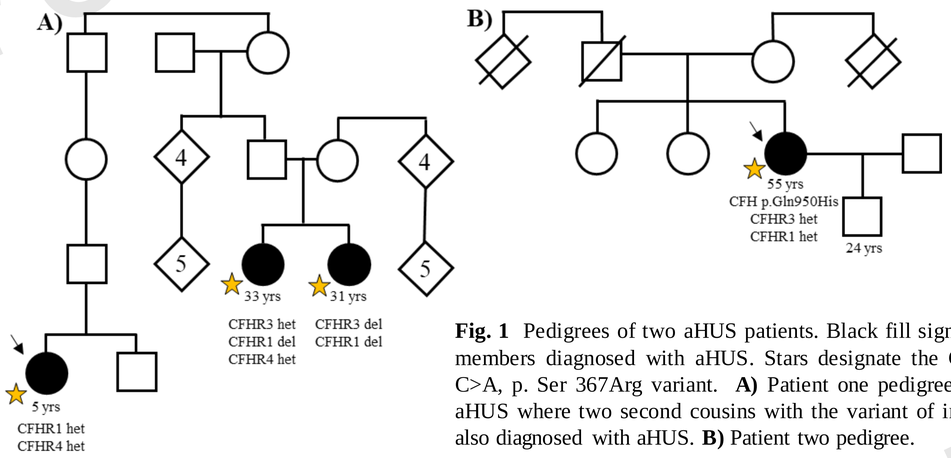

Fig. 1 Pedigrees of two aHUS patients. Black fill signifies family members diagnosed with aHUS. Stars designate the CFB c.1101 C>A, p. Ser 367Arg variant. **A)** Patient one pedigree of familial aHUS where two second cousins with the variant of interest were also diagnosed with aHUS. **B)** Patient two pedigree.

Results

Genetic analysis revealed a novel heterozygous variant in *CFB* (c.1101 C>A, p.Ser367Arg). This variant lies within the von Willebrand Factor type A (vWFA) domain of the catalytic Bb subunit of fB. Previous experimental data demonstrates an association between variants in the vWFA domain and promotion of high-affinity C3 binding.

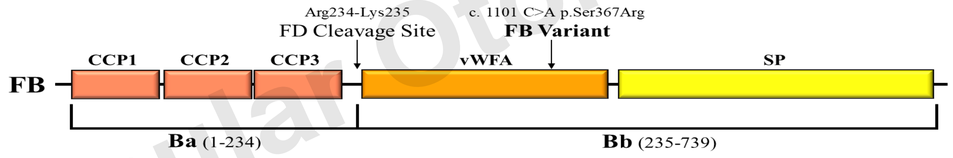

Fig. 2. Factor B consists of five domains which make up two subunits. The N terminus is connected to three complement control protein (CCP) domains -- which comprise the Ba subunit – a von Willebrand type A (vWA) domain and a serine protease (SP) domain – which make up the catalytic Bb subunit and contains the variant of interest.

Assay (normal range)	**Patient 1**	**Patient 2**
APFA (50-130%)	0%	0%
CH50 (30-90 AU)	13	14
Hemolytic (< 3%)	0%	0.40%
IFE (< 7.5%)	4 + (76.3%)	4+ (71.6%)
FBAA (< 200 AU)	< 50	< 50
FHAA (< 200 AU)	< 50	< 50
C3 Level (0.9-1.8 g/L)	< 0.16	< 0.16
C4 Level (9.4-51.4 mg/dL)	0.22	0.30
FB Level (22-50 mg/dL)	41.5	36.2
Ba Level (<1.2mg/L)	1.50	3.77
Bb Level (<2.2 mg/L)	4.20	1.98
Properdin (10-33 mg/L)	5.8	12.4
sC5b-9 (<0.3 mg/L)	0.92	0.34
FH Level (180-420 mg/L)	183	323

Table 1. Functional biomarkers for patient one and two show a pattern of complement dysregulation. Similarly, both patients, have low levels (grey) of alternative and complement pathway activity as well as low circulating C3 and C4 levels. Patient one also had low levels of Properdin. Patients had high results (red) for IFE and activation products (soluble C5b-9 and Ba or Bb). While within the normal range, FH level varies significantly between patients.

- Although the factor B level was normal for both patients, an increased binding affinity of fB to C3 corresponds to biomarker data, which is consistent with AP over-activity leading to protein depletion.

Modified Immunofixation Electrophoresis (IFE)

- Based on a lack of autoantibodies and paraproteins, patient immunoglobulin G (IgG) was depleted and purified to determine the effects of patient IgG on convertase stability.

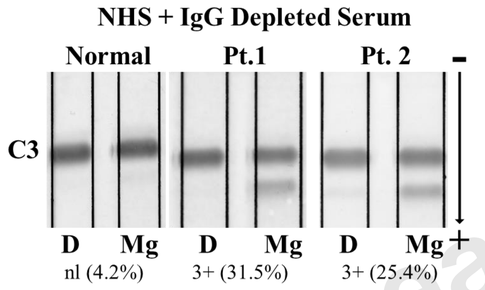

Fig. 3 IgG depleted patient samples combined with normal human serum remained positive, indicating a portion of C3 breakdown independent of IgG activity. In this case, breakdown is presumed to be due to the fB variant. Additionally, for patient two, IFE was also positive (39.6%) when using purified IgG and normal human serum. This suggests patient two IgG may play a role in classical pathway activation or C5 convertase formation

C3 deposition

- When testing only patient serum, there was no C3 deposition in either patient. To assess if this was due to the depletion of necessary proteins, such as C3, three modifications were created: mixing normal human serum with patient serum, spiking C3 protein, and spiking C3 and fH protein into patient serum.

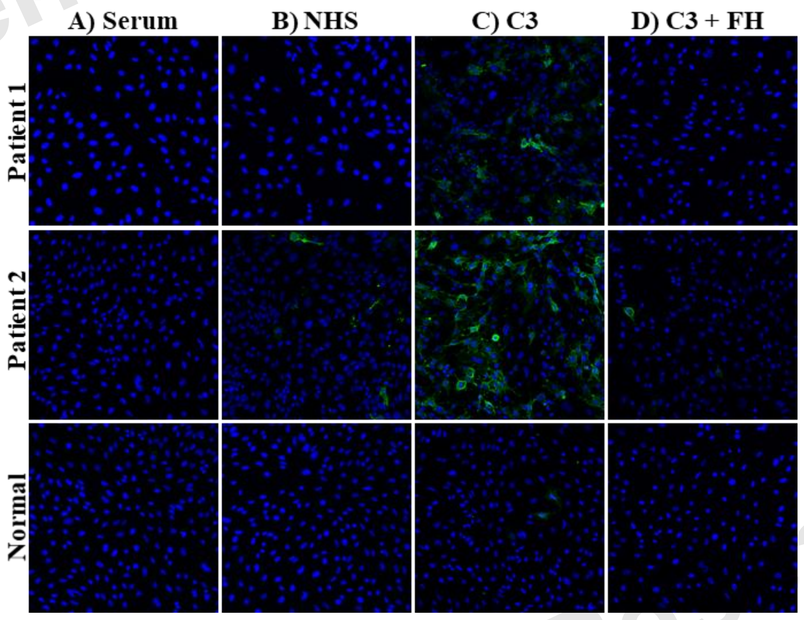

Fig. 4 A) Deposition of C3 is minimal when using patient serum. **B)** A mixture of normal human serum (NHS) and patient serum resulted in a slight increase in C3 deposition in pt. 2, but not in pt. 1. **C)** When 250ng/µL C3 is added to patient samples, there is a large increase in C3 deposition in both patients compared to the normal control. This suggests the patient C3 protein depletion limits complement activation. D) When 250ng/µL C3 and 50ng/µL fH are added, C3 deposition decreases. Therefore adding C3 to patient sample enabled more effective progression through the complement cascade than in a healthy individual, indicating differences in activation or regulatory processes. The addition of purified fH protein limited this over-activation.

Sheep Cell Decay Assay

- In order to assess the implications of the variant, a S367R mutant fB protein and a wild-type fB protein were produced and purified.
- Normalized proteins were combined with sheep erythrocytes, rat serum, and factor D (fD) to form the C3 convertase. Lysis was then monitored over time in order to assess convertase activity.

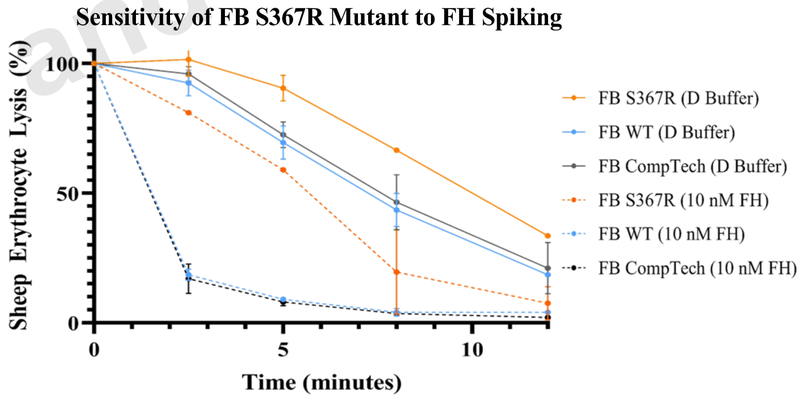

Fig. 5. Sheep erythrocyte lysis was monitored in the presence of wild type and mutant S367R FB. In D buffer conditions, The fB S367R mutant showed increased stability compared to wild type fB and commercial fB. Additionally, when fH was spiked in, the fB S367R mutant showed resistance to regulation when compared to wild type and commercial fB spiked with fH.

Conclusion

In conclusion, this data suggests fB p.Ser367Arg is a gain-of-function mutation that increases fB binding-affinity, which leads to consumption of C3 and increased C3 convertase stability. In the two patients described, this level of fluid phase AP dysregulation lead to early-onset aHUS. This case highlights the importance of understanding the functional consequence of each variant and provides insight into the complexities of the pathogenesis of aHUS.

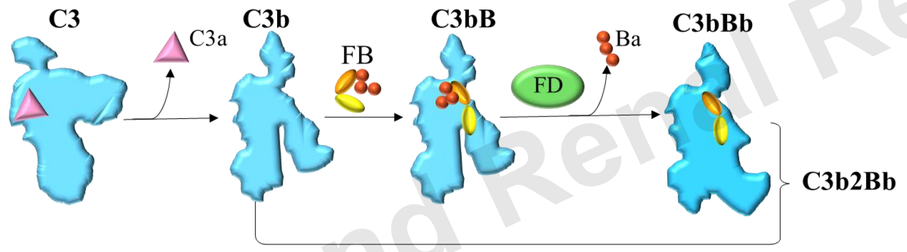

Fig. 6. Formation of the C3 and C5 convertase is highly dependent on fB activation. An increase in fB binding affinity to C3b drastically increases convertase formation and therefore C3 consumption. Once formed, high affinity fB mutants are more resistant to decay, thus increasing the half life of the unstable C3 convertase. Increased convertase stability results in a more effective amplification of C3b and progression through the complement cascade.

Acknowledgement

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