The Functional Consequence of two *CFI* Ultra Rare Variants in Complement-Mediated Diseases: Insights from an *In Vitro* Splicing Assay

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Introduction

The complement cascade is activated via one of three pathways: classical, lectin, or alternative. Its activation converges at the cleavage of C3 into C3a and C3b. C3b can self-amplify or trigger activation of the terminal pathway and lead to the formation of the membrane attack complex (MAC, C5b-9). The complement cascade is an essential component of the innate immune system, however, over activation of the complement cascade can result in various complement-mediated diseases.

Complement Factor I (FI) is an important serine protease that directly regulates complement activity by inactivating C3b and C4b. Variants in *cis*-acting elements in *CFI* can disrupt alternative splicing and result in low expression of FI protein. Consequently, reduced FI can lead to the accumulation of C3b deposits on the cell surface and potentially drive the pathogenicity of complement-mediated diseases such as C3 glomerulopathy (C3G), atypical hemolytic uremic syndrome (aHUS), or various forms of recurrent infections.

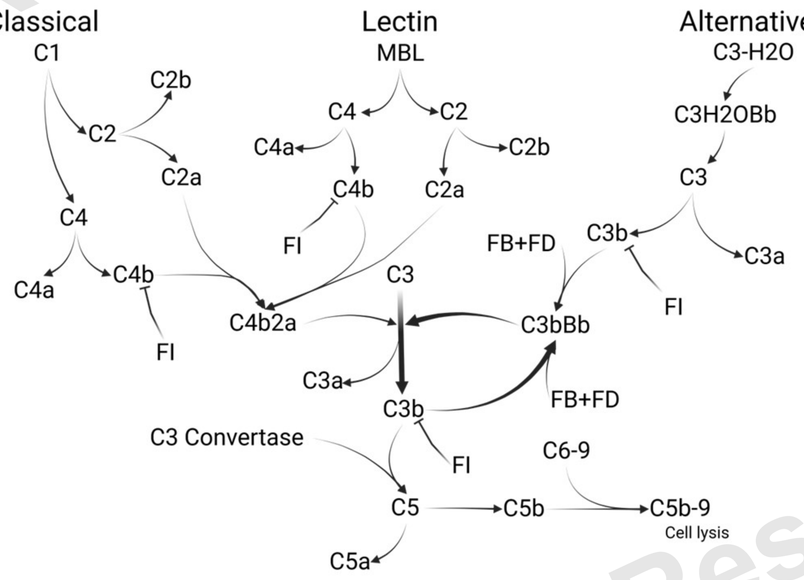

Figure 1. **Complement Cascade.** Complement is activated via the classical, lectin, and alternative pathways (AP). The activation converges at the cleavage of C3 to C3b, where the latter can enter the amplification loop of the AP or progress along the terminal pathway. FI inactivates C4b and C3b and prevents them from continuing in their respective pathway.

Methods

1. Identify patients with *CFI* variants in our cohort of C3G and/or aHUS
2. Determine FI expression by ELISA and Western Blot
3. Clone DNA fragment into the pET01 plasmid
4. Transfect the plasmid into a mammalian cell line
5. Isolate transcribed RNA and preform RT-PCR
6. Calculate the amount of each transcripts synthesized using Image J
7. Preform Sanger sequencing

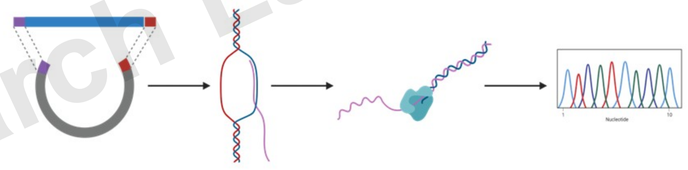

Figure 2. **Experimental design.** Variants in *CFI* were amplified and cloned into the pET01 plasmid via a recombination method. After transfection in mammalian cells, the transcribed RNA was isolated and reverse transcribed into cDNA. The cDNA was amplified, sequenced and analyzed for alternative splicing.

Results

Table 1. **Patients and Variants.**

Patient	Sex	Variant	FI Levels (mg/L)*	Zygosity	Diagnosis
Pt 1	F	c.1429G>C	16.8	Het	aHUS
Pt 2	F	c.1429G>C	26	Het	C3GN/C3G
Pt 3	M	c.1429G>C	15.8	Het	aHUS
Pt 4	F	c.1429+1G>C	Undetectable	Hom	Recurrent infection
Pt 5	M	c.1429+1G>C	Not Available	Het	C3GN/C3G

*Normal FI Levels: 18-44 mg/L

Table 2. **Variant information.**

Variant	Genomic Position	Allele Count*	Allele Number*	MAF*	Splice Score**
c.1429G>C	4-110667378-C-G	5	251250	1.99E-5	Broken WT Donor Site (71.94)
c.1429+1G>C	4-110667377-C-G	8	282668	2.83E-5	Broken WT Donor Site (55.96)

*collected from the gnomAD database (GRCh37/hg19; ENSG00000205403.8).
**collected from Human Splice Finder

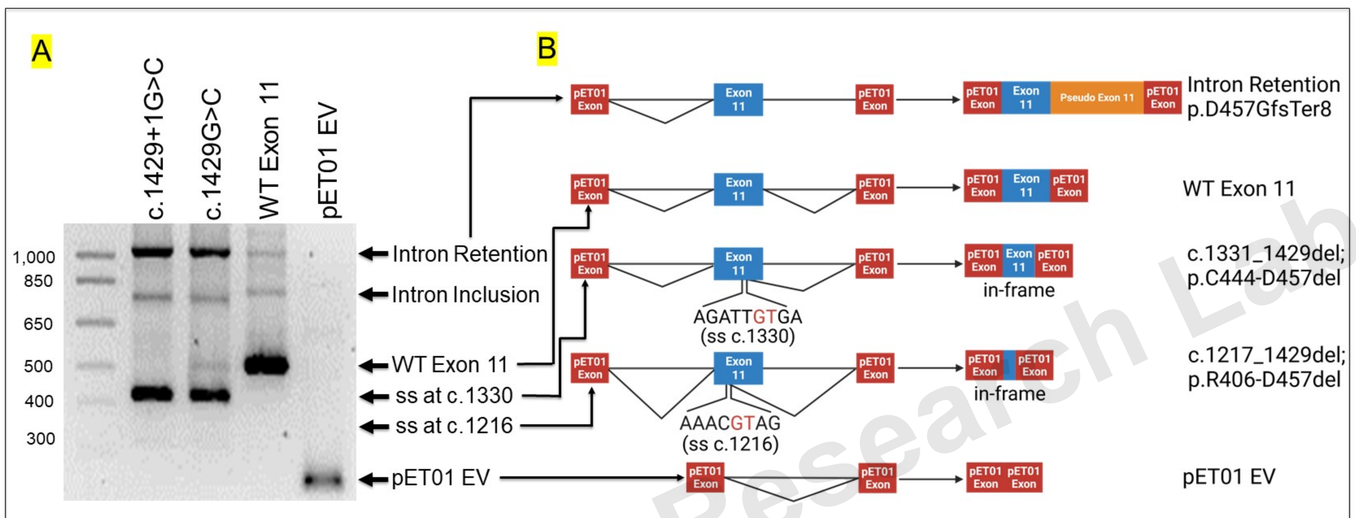

Figure 4. **Variants affects on RNA splicing. (A)** RT-PCR results. A 2% agarose gel depicting the impact of variants (c.1429G>C and c.1429+1G>C) on RNA splicing. Compared to the controls with wild type (WT) Exon 11, both variants create multiple aberrant alternative splicing forms. pET01 Empty Vector (EV) serves as a negative control. **(B)** Visual depictions show Sanger sequencing results, arranged from the uppermost to the lowermost band. The anticipated protein outcomes are listed on the right-hand side.

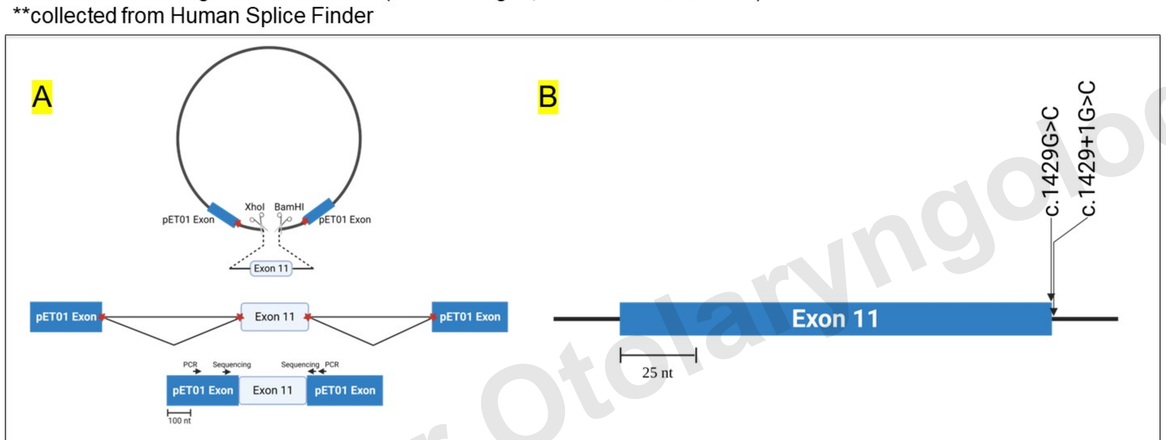

Figure 3. **Exon 11 Construct. (A)** Exon 11 in *CFI* was cloned into the pET01 plasmid using *Cre* recombinase. The plasmid was transformed into *E. coli* for plasmid amplification and then transfected into mammalian cells where splicing occurred. **(B)** c.1429+1G>C is an intronic variant that changes the canonical splice donor site while c.1429G>C is a variant of unknown significance at the end of exon 11.

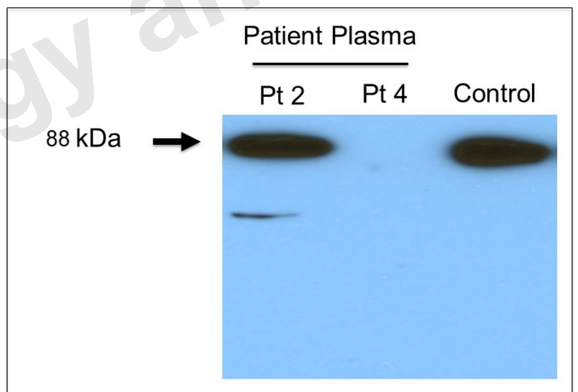

Figure 5. **Western Blots.** A non-reducing gel (4-15%) was blotted with primary antibody (FI EPR23948-48) that is specific to the heavy chain. Pt 4 carries c.1429+1G>C (hom) while Pt 2 is a heterozygote for c.1429G>C.

Table 3. **Quantitative Analysis of Transcript Abundance.**

Splicing Event	c.1429+1G>C	c.1429G>C	WT Exon 11
Intron Retention	34%	28%	4%
Intron Inclusion	11%	10%	7%
WT Exon 11	0%	10%	89%
c.1330	54%	51%	0%
c.1216	1%	1%	0%
Total	100%	100%	100%

The splicing events correspond to those of **Figure 4**.

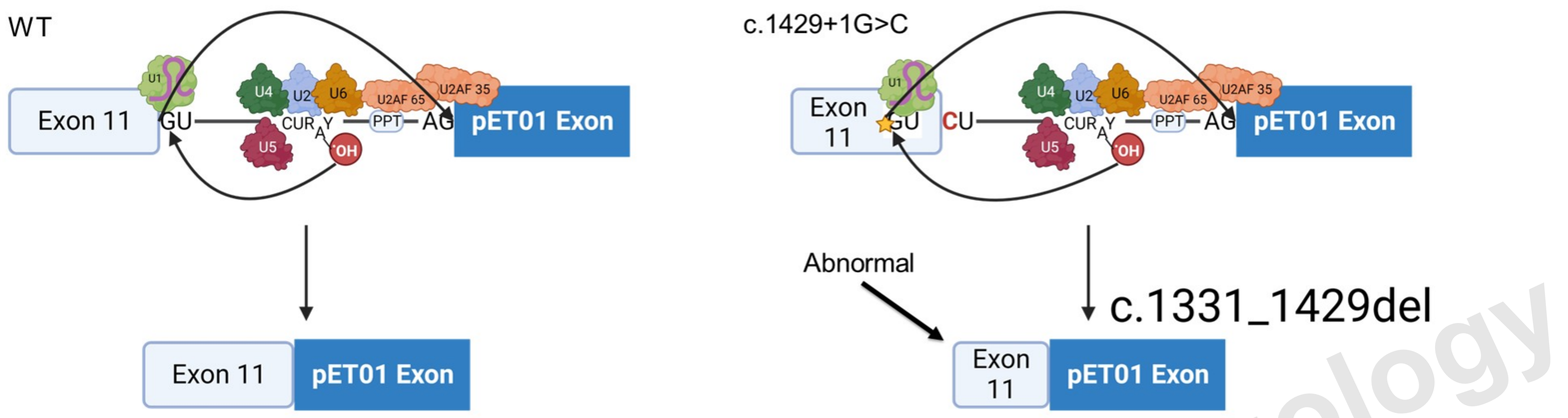

Figure 6. **Splicing Mechanism for Minigene.** Left is WT exon 11, while the right is exon 11 and the canonical splice donor mutation (shown in red). The consequence of the splice mutation is recognition of a cryptic splice donor site near the end of exon 11. When the cryptic splice site is used, the product is a truncated exon 11 (bottom right) which was resolved by agarose gel and Sanger sequencing (Figure 4).

Conclusion

This study shows that the missense mutation in *CFI,* c.1429G>C, has a substantial impact on pre-mRNA splicing. The c.1429G>C change leads to activation of cryptic splicing donor sites within exon 11 and results in haploinsufficiency. This finding is mirrored by the changes seen secondary to alteration of the neighboring highly conserved splicing donor site variant c.1429+1G>C. These findings highlight the importance of functional studies to understand the impact of rare genetic variants in *CFI* in patients with complement-mediated diseases.

Acknowledgements

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