

Molecular diagnosis of haemophilia A: four novel variants identified in five patients

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Introduction:

Haemophilia A (HMA) is an X-linked bleeding disorder caused by reduced levels of the coagulation factor VIII (FVIII) due to alterations in the *F8* gene. Decreased levels of FVIII coagulant activity (FVIII:C) leads to a loss of clotting activity and consequent bleeding (predominantly into joints, muscles and inner organs). The severity of HMA ranges from mild (5-30% FVIII:C) to moderate (2-5% FVIII:C) to severe (<1% FVIII:C). During the last five years, we have found four novel variants identified in five index patients with no family history of HMA.

Results:

F8 variant analysis allowed identification of three frameshift and one missense variants: c.1060_1061delCT, c.3561dupT and c.4804delC detected in families presenting severe HMA; c.5836G>T variant was identified in two unrelated patients with a mild phenotype (Table 1; Figures 1-4). None of these variants had been previously reported.

Methodology:

Analysis of the *F8* gene was performed in five index patients (one female from a family without previous molecular studies, index case not available), using genomic DNA extracted from peripheral EDTA blood samples and specific PCR for *F8* exons, followed by Sanger sequencing. *F8* IVS22 and IVS1 inversions were excluded in severe HMA cases. Bioinformatics analysis was performed with several pathogenicity prediction tools (Alamut Visual, VarSome, VEP and Human Splicing Finder).

Table 1. Novel *F8* variants identified.

Patient	Variant type	HMA clinical phenotype	<i>F8</i> location	Nucleotide change (NM_000132.3)	Amino acid change (NP_000123.1)
P1	Missense	Mild	Exon 18	c.5836G>T (Fig. 1)	p.(Asp1946Tyr)
P2					
P3	Frameshift	Severe	Exon 8	c.1060_1061delCT (Fig.2)	p.(Leu354Thrfs*5)
P4			Exon 14	c.3561dupT (Fig.3)	p.(Pro1188Serfs*10)
P5				c.4804delC (Fig.4)	p.(Gln1602Lysfs*19)

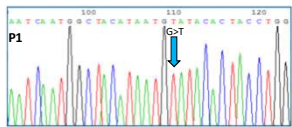
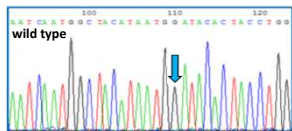


Figure 1. Electropherogram obtained from direct nucleotide sequencing of *F8* exon 18 (forward primer) showing *F8* c.5836G>T p.(Asp1946Tyr) variant.

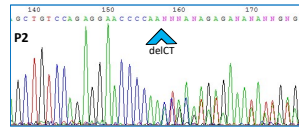
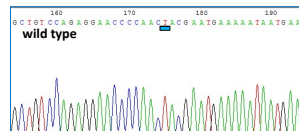


Figure 2. Electropherogram obtained from direct nucleotide sequencing of *F8* exon 8 (forward primer) showing *F8* c.1060_1061delCT p.(Leu354Thrfs*5) variant. Results from a female carrier.

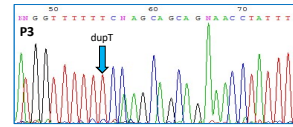
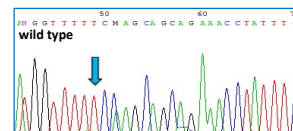


Figure 3. Electropherogram obtained from direct nucleotide sequencing of *F8* exon 14 (forward primer) showing *F8* c.3561dupT p.(Pro1188Serfs*10) variant.

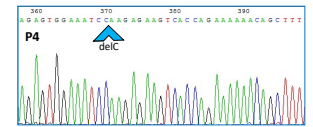
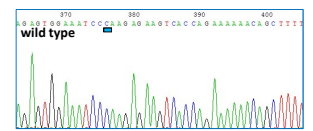


Figure 4. Electropherogram obtained from direct nucleotide sequencing of *F8* exon 14 (forward primer) showing c.4804delC p.(Gln1602Lysfs*19) variant.



Figure 5. Splicing effect predicted by Human Splicing Finder for the novel *F8* variant c.5836G>T, p.(Asp1946Tyr)

Potential Splice Sites		Potential Branch Points		Enhancer motifs		Silencer motifs		Other splicing motifs	
Sequence Position	cDNA Position	Splice site type	Motif	New splice site	Wild Type	Mutant	If cryptic site use, exon length variation	Variation (%)	
117	17	Donor	AATGGATC	AATgtatc	382	65.03	-28	100.00	

Discussion:

In the three patients with severe HMA, three different novel *F8* variants were identified: c.1060_1061delCT, p.(Leu354Thrfs*5) (Fig. 2), c.3561dupT, p.(Pro1188Serfs*10) (Fig. 3) and c.4804delC, p.(Gln1602Lysfs*19) (Fig. 4). All these variants create a frameshift, leading to a premature termination codon and presumably resulting in non-functional truncated proteins, confirming the patient's phenotypes.

The novel *F8* missense variant c.5836G>T, p.(Asp1946Tyr) (Fig. 1) was identified in two unrelated patients, both with mild HMA. The Asp1946 is a highly conserved amino acid in the FVIII protein. Additionally, physicochemical properties between Asp and Tyr are significantly different (while Asp is a small, negatively charged, and polar, Tyr is an hydrophobic, aromatic amino acid) and *in silico* analysis classified it as pathogenic due to the amino acid substitution. According to *in silico* analysis, this variant can also disturb the normal mRNA splicing process due to the creation of a new donor splice site (Figure 5). RNA studies and other functional assays are essential in order to establish this variant clinical significance.

Identification of novel pathogenic *F8* variants in HMA patients allows genotype-phenotype correlations, appropriate genetic counseling and new knowledge about the molecular bases of this pathology.

REFERENCES:

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- Varsome The Human Genomics Community - <https://varsome.com>
- <http://www.hgvs.org/mutnomen>;
- Ensembl Variant Effect Predictor (VEP) - <https://www.ensembl.org> vep;
- Human Splicing Finder - Version 3.1 - www.umd.be