Polymorphic variation within the *VWF* gene contributes to the failure to detect mutations in patients historically diagnosed with type 1 von Willebrand disease from the MCMDM-1VWD cohort

Type 1 von Willebrand disease (VWD) results from a partial quantitative deficiency of plasma von Willebrand factor (VWF). Three multicenter studies were conducted to elucidate the molecular and clinical features of type 1 VWD; undertaken in the European Union (EU), Canada and the UK.[1-3] All three successfully identified *VWF* genetic alterations likely to cause type 1 VWD, but failed to identify a genetic cause in between 30% and 40% of patients.

In the EU study, MCMDM-1VWD, the genetic cause remained unknown in 46 of 150 index cases (IC).[2] Linkage analysis[4] indicated complete co-segregation between *VWF* and a type 1 VWD phenotype in 10 of 46 (22%) of these mutation-negative IC, suggesting a genetic defect in *VWF* may have been overlooked. Additionally, 18 of 46 (39%) were uninformative for linkage; a *VWF* defect in this group could not be discounted. The *VWF* locus is highly polymorphic containing more than 1,100 single nucleotide polymorphisms (SNP), with over 150 unique SNP located within exons and closely flanking intronic sequence. Recently, a VWD patient was reported in whom a heterozygous *VWF* mutation was initially missed due to occurrence of SNP c.4641T>C (rs216310) within the annealing site of a primer used to amplify exon 28 of the patient's DNA.[5] SNP c.3675-75A>G (rs216312) in intron 27 has similarly been reported to affect *VWF* mutational analysis.[6,7] In the EU study, this SNP caused a heterozygous IC and affected relatives to appear homozygous for the c.3943C>T (p.Arg1315Cys) mutation.

When all three type 1 VWD studies were initiated, *VWF* genomic sequence information was incomplete. Designed PCR primers were based primarily on partial *VWF* sequence, greatly restricting primer annealing site location. This study aimed to investigate the extent to which heterozygous SNP occurred within annealing sites of EU study primers and to ascertain whether SNP had caused heterozygous genetic defects in *VWF* to have been missed due to mono-allelic amplification of only the wild-type *VWF* allele.

Twenty-eight mutation-negative EU study IC (historically diagnosed with type 1 VWD[2]) were investigated, ten demonstrating complete co-segregation of disease phenotype with *VWF*,[4] the remainder from small families of 3 or less individuals (non-informative for linkage). Genomic DNA was available for IC, their affected (AFM) and unaffected (UFM) family members, and from healthy controls (HC). Extensive phenotypic data were available for the majority of subjects.[2]

SNP within *VWF* reported on dbSNP (build 130) and/or the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee on VWF online database (VWFdb; http://*www.vwf.group.shef.ac.uk/*), and EU study primer annealing sites[2] were mapped to a *VWF* reference sequence (NCBI build 37.1 NC_000012.10; g.5925659-6107623). Re-designed primers were amplified under standard PCR conditions and amplicons analyzed using direct DNA sequencing. Sequences were compared against the *VWF* reference sequence using the Staden Package.[8] Primer sequences and reaction conditions are available on request. The effect of intronic sequence alterations was determined using six splice-site prediction tools.[9]

Table 1. Genotype and phenotype in historically diagnosed type 1 VWD families with previously unreported mutations.

Family	Subject	Status	Candidate mutation(s)[1]		VWF:Ag (IU/dL)	VWF:RCo (IU/dL)	FVIII:C (IU/dL)	VWF:FVIIIB slope[2]	ABO blood group	Bleeding score[3]	AbM[4]
			Allele 1	Allele 2							
P6F11	II1	IC	c.3675-14G>A	c.2771G>A (p.Arg924Gln)	40	34	52	1.08	O/O	19	No
	II4	AFM	c.2771G>A (p.Arg924Gln)	c.2771G>A (p.Arg924Gln)	49	50	71	ND[6]	O/O	3	No
	III1	AFM	c.2771G>A (p.Arg924Gln)	-	62	52	68	1.22	O/O	3	No
	II3	UFM	c.2771G>A (p.Arg924Gln)	-	64	69	77	ND	O/O	2	No
	III2	UFM	c.3675-14G>A	-	84	82	96	ND	O/A	3	No
P6F14	III1	IC	c.3179G>A (p.Cys1060Tyr)	-	36	28	39	0.44	O/O	9	2A(IIE)
	II1	AFM	c.3179G>A (p.Cys1060Tyr)	-	39	39	32	0.45	O/O	5	2A(IIE)
P7F5	II4	IC	c.5842+5G>A	-	72	77	86	1.13	O/A	-1	2A(unspecified)
	I1	AFM	c.5842+5G>A	c.2435delC (p.Pro812ArgfsX31)	5	3	26	1.54	O/A	18	2A(unspecified)[5]
	II1	AFM	c.5842+5G>A	-	79	58	199	1.07	O/A	4	2A(unspecified)
	III1	AFM	c.5842+5G>A	-	39	34	72	0.99	O/O	2	2A(unspecified)
	III2	AFM	c.5842+5G>A	-	46	49	82	1.11	O/A	2	2A(unspecified)

[1]Mutations p.Pro812ArgfsX31 and p.Arg924Gln previously identified using original primers. [2]Normal range in HC 0.68-1.44. [3]Based on the MCMDM-1VWD bleeding questionnaire, a score ≥4 indicative of 'excessive' bleeding.[2] [4]Based on detailed multimer analysis.[2,13] [5]AbM profile more severe compared to other AFM profiles observed within the family. [6]ND = not determined.

Table 2. Novel SNP identified during re-analysis of historically diagnosed type 1 VWD families.

Variant	Location	Frequency[1]	Predicted effect on splicing	Reason variant previously unreported
c.3109-66C>T	Intron 23	0.996/0.004	None	Identified in HC while screening for p.Cys1060Tyr mutation
c.3223-102C>T	Intron 24	0.996/0.004	Disrupts potential SFRS1[3] splicing enhancer binding site	Outside region originally analyzed
c.3223-265T>G	Intron 24	0.875/0.125	None	Outside region originally analyzed
c.4146G>T (p.Leu1382Leu)	Exon 28	0.996/0.004	Strengthens cryptic acceptor site at c.4160[2]	Sample previously not screened

*[1]Based on presence in 28 IC and 100 HC. [2]*In silico *analysis indicates wild-type acceptor site remains 73% stronger. [3]SFRS1 = serine/arginine-rich splicing factor 1.*

The EU study[2] previously utilized five different primer sets. On average, each set contained primers for 15 of 52 exons where SNP were identified within primer annealing sites; primers with SNP identified within the 3' nine nucleotides were re-designed (18 primer pairs). Re-analysis identified three novel candidate mutations (c.3179G>A (p.Cys1060Tyr), c.3675-14G>A, and c.5842+5G>A). All were inherited in *cis* with an SNP within an original primer annealing site (c.3222+31C>T (rs73051263; intron 24), c.3675-75A>G (rs216312; intron 27), and c.5665-118A>G (rs216305; intron 33), respectively) and were not observed in 100 population-matched HC. Complete co-segregation of disease phenotype with *VWF* was observed in two families. Notably, in each instance the SNP was located within the 3' four nucleotides of the primer annealing site. Genotypic and phenotypic data are summarized in Table 1.

Cys1060 forms an intra-chain disulphide bond with Cys1084[10] highlighting a key structural/functional importance. Substitution of cysteine by tyrosine is predicted to disrupt this disulphide bridge, correlating with observed abnormal multimers (AbM; Table 1). The change also results in a heterozygous VWF:FVIIIB reduction (0.44-0.45) in the IC and AFM (HC normal range 0.68-1.44), highlighting p.Cys1060Tyr as a type 2N VWD mutation, symptomatic in the heterozygous state. The previously described type 2N VWD mutation p.Cys1060Arg, in compound heterozygosity with p.Arg924Gln, also results in AbM and mild reduction in VWF:FVIIIB,[11] further supporting the p.Cys1060Tyr observations.

c.5842+5G>A, which was also associated with AbM, was predicted by all six *in silico* splice-site tools to completely abolish the exon 34 splice donor site. This could result in either exon skipping or intron retention, both predicting a frameshift (p.Pro1889AlafsX12 and p.Val1949GlufsX17, respectively). Patient mRNA or *in vitro* analysis may help verify the *in silico* prediction. A frameshift would disrupt intra-chain disulphide bond formation after the VWF D4 domain[10] in turn affecting multimerization. Furthermore, the D4-CK region acts as an initial binding site for ADAMTS13 prior to VWF A2 domain cleavage,[12] so premature VWF termination after the D4 domain could influence proteolysis by ADAMTS13.

c.3675-14G>A was predicted *in silico* to have little effect on splicing efficiency. It was identified both in the IC and in an UFM with normal VWF levels (Table 1) and did not segregate with disease phenotype, suggesting that another mutation remains to be identified within this family. *VWF* re-analysis also highlighted additional rare sequence variation previously unreported on either dbSNP or VWFdb (Table 2).

p.Cys1060Tyr and c.5842+5G>A were both associated with AbM, but still considered type 1 VWD mutations according to the 2006 VWD classification.[13] Fifty-seven of 150 EU study IC had minor multimer abnormalities.[2,13] AbM correlate well with the identification of mutations, the results from this study bringing the total to 56 of 57 (98%). Analysis from the Canadian type 1 VWD study supports this observation, where 28 of 29 IC with AbM

have an identified *VWF* mutation.[14] Multimer abnormalities are, therefore, indicative of a *VWF* mutation being present.

In conclusion, SNP within primer annealing sites complicate molecular analysis of VWD, specifically detection of heterozygous mutations. This would apply to all *VWF* molecular analysis, potentially including both the Canadian and UK type 1 studies. Primer design for *VWF* should, therefore, be reviewed on a regular basis.

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