Five novel mutations in the gene for human blood coagulation factor V associated with type I factor V deficiency

Richard van Wijk, Karel Nieuwenhuis, Marijke van den Berg, Eric G. Huizinga, Brenda B. van der Meijden, Rob J. Kraaijenhagen, and Wouter W. van Solinge

Coagulation factor V (FV) plays an important role in maintaining the hemostatic balance in both the formation of thrombin in the procoagulant pathway as well as in the protein C anticoagulant pathway. FV deficiency is a rare bleeding disorder with variable phenotypic expression. Little is known about the molecular basis underlying this disease. This study identified 5 novel mutations associated with FV deficiency in 3 patients with severe FV deficiency but different clinical expression and 2 unaffected carriers. Four mutations led to a premature termination codon either by a nonsense mutation (single-letter amino acid codes): A1102T, K310Term. (FV Amersfoort) and C2491T, Q773Term. (FV Casablanca) or a frameshift: an 8–base pair deletion between nucleotides 1130 and 1139 (FV Seoul$_1$) and a 1–base pair deletion between nucleotides 4291 and 4294 (FV Utrecht). One mutation was a novel missense mutation: T1927C, C585R (FV Nijkerk), resulting in the absence of mutant protein despite normal transcription to RNA. Most likely, an arginine at this position disrupts the hydrophobic interior of the FV A2 domain. The sixth detected mutation was a previously reported missense mutation: A5279G, Y1702C (FV Seoul$_2$). In all cases, the presence of the mutation was associated with type I FV deficiency. Identifying the molecular basis of mutations underlying this rare coagulation disorder will help to obtain more insight into the mechanisms involved in the variable clinical phenotype of patients with FV deficiency. (Blood. 2001;98:358-367)

© 2001 by The American Society of Hematology

Introduction

Human coagulation factor V (FV) is a single-chain glycoprotein that plays an important role in maintaining the hemostatic balance. It circulates in blood as an inactive procoagulant with a M_r of 330 kd and a structure consisting of 3 homologous A-type domains and 2 homologous C-type domains connected by a heavily glycosylated B domain in the order A1-A2-B-A3-C1-C2. Proteolytic cleavage by thrombin at R709, R1018, and R1545 (single-letter amino acid codes) results in removal of the B domain and converts the procofactor into the fully active cofactor FVa, which consists of a M_r 105-kd heavy chain (A1-A2) and a M_r 74- or 71-kd light chain (A3-C1-C2), associated via a single Ca^{++} ion.[1-3] The difference in molecular weight of the light chain reflects the presence of 2 isoforms of FVa (FVa$_1$ and FVa$_2$) due to alternative glycosylation of the C2 domain, which leads to different affinities for biologic membranes and subsequent overall procoagulant activity.[4,5] In its active form, FVa forms an essential part of the prothrombinase complex that catalyzes the conversion of prothrombin to thrombin by factor Xa in the presence of calcium and a phospholipid membrane.[1-3] Activated protein C (APC) inactivates FVa through cleavage of the active cofactor at R306, R506, and R679 and requires FV as a cofactor in the APC-mediated inactivation of factor VIIIa (FVIIIa).[6,7] Thus, FV plays an important role in the procoagulant pathway as well as in the protein C anticoagulant pathway. The structure of FV is similar to FVIII (both cofactors share approximately 40% homology in their heavy and light chains) and ceruloplasmin, the copper-binding protein in plasma.[8,9]

Recently, the crystal structure of the C2 domain of FV has been established[10] and molecular models for the A and C domains of FV have been proposed.[11,12]

The gene for coagulation FV has been mapped to chromosome 1q23[13] and spans more than 80 kilobases (kb). It consists of 25 exons and the messenger RNA (mRNA) encodes a leader peptide of 28 amino acids and a mature protein of 2196 amino acids. Roughly, the heavy chain is encoded by exons 1 to 12 and the light chain by exons 14 to 25. The entire B domain is encoded by exon 13, which contains 2 tandem repeats of 17 amino acids and 31 tandem repeats of 9 amino acids that are absent in the B domain of FVIII.[14,15]

Deficiency of FV, or parahemophilia, was first described in 1947 by Owren.[16] It is a rare autosomal recessive bleeding disorder with an estimated frequency of one in one million. The phenotypic expression of FV deficiency is variable; heterozygotes are usually asymptomatic, whereas homozygous patients show mild, moderate, or severe bleeding symptoms. Identifying the molecular basis underlying this disease will help to obtain more insight into the mechanisms involved in this variable clinical expression. The recently published complete nucleotide sequence of the *FV* gene (GenBank accession number Z99572) has facilitated the molecular characterization underlying FV deficiency and reports have identified mutations in the *FV* gene that result in FV deficiency.[17-30] To date, no compound heterozygous patients have been described. In the present study we used DNA sequence analysis to detect 5 novel

mutations and one previously reported mutation in the *FV* gene of 3 patients, one of whom is the first characterized compound heterozygote, with severe FV deficiency and 2 asymptomatic carriers. All mutations resulted in type I FV deficiency.

Patients, materials, and methods

Patients

Patient 1 is a 19-year-old woman from South Korea. She was adopted by a Dutch family at the age of 3 months. At the age of 19 months she developed bleeding of the soft tissue of the mouth. Severe FV deficiency, reflected by an FV activity less than 1% (Table 1), was diagnosed. At the age of 4 years she experienced a large subdural hematoma, which completely resolved after frequent transfusions of fresh frozen plasma. Her bleeding pattern mostly showed soft tissue bleeds of the mouth, epistaxis, and hematomas for which she received fresh frozen plasma once every 3 months. In the last years her bleeding pattern changed to spontaneous muscle bleedings.

Patient 2 is a 5-year-old boy of Turkish ancestry. He was diagnosed at the age of 1 year, because of soft tissue bleed in his mouth that did not stop after conservative treatment. His laboratory tests demonstrated an FV activity less than 1%. His consanguineous parents both had lowered FV activity and antigen levels, suggesting a FV deficiency carrier status (Table 1). No clinical bleeding problems in the parents were apparent. The following years he frequently had bleeds of his mouth, hematomas, and epistaxis. Severe bleeding episodes were treated with fresh frozen plasma. Until now he has not had muscle or joint bleeds.

Patient 3 is a 15-year-old girl from Morocco. The diagnosis of severe FV deficiency, FV activity less than 1% (Table 1), was made following family screening. She does not have a bleeding tendency, but so far she has not had any operative procedures or injuries. Her parents are first-degree cousins. Her 23-year-old brother also has a severe FV deficiency. Other than prolonged bleeding after injuries, he has had no bleeding problems. He has been described before.[31]

Patients 4 and 5 are both asymptomatic Dutch individuals who were discovered during routine laboratory testing. They presented with normal to slightly prolonged activated partial thromboplastin time (APTT), respectively, 31 and 34 seconds (reference values, 24-32 seconds), and slightly prolonged prothrombin time (PT), respectively, 13.6 and 13.8 seconds (reference values, 11.0-13.0 seconds). Liver failure and all other possible causes of aberrant APTT and PT results were excluded. FV activity and antigen levels were decreased (Table 1).

Informed consent was obtained from all patients and family members.

Control group

The control group consisted of 50 unrelated healthy laboratory employees of Caucasian origin. Their DNA was used to establish the allelic frequencies of exonic polymorphisms and the occurrence of the T1927C missense mutation by testing the appropriate base change either by restriction enzyme analysis or by DNA sequence analysis. Informed consent was obtained from all control individuals.

Blood collection for coagulation tests

Venous blood was collected into plastic tubes in 1/10 volume of 3.8% sodium citrate. Platelet-poor plasma was obtained by centrifugation at 1200*g* for 20 minutes at room temperature and aliquots were immediately stored at −80°C until testing.

FV coagulant activity and FV antigen

Activity of FV was measured on an STA coagulation analyzer (Roche, Mannheim, Germany) by performing a PT in a plasma sample diluted with FV-deficient plasma. FV antigen was determined by Professor Rogier M. Bertina by measuring FV light chain by an enzyme-linked immunosorbent assay (ELISA) as described.[20]

Isolation of DNA and RNA

DNA was isolated from peripheral white blood cells using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Lymphocytes were isolated from whole blood using Ficoll-Pague (Pharmacia, Uppsala, Sweden) and total RNA was extracted as described.[32]

Amplification of the *FV* gene

Nucleotides are numbered according to Jenny and colleagues.[14] Nucleotides in the putative promoter region are numbered relative to the initiation codon. The primers used for polymerase chain reaction (PCR) amplification

Table 1. FV activity and antigen data from the families described in this study

	FV mutation	Genotype*	FV activity (%)	FV antigen (%)
Normal plasma	—	—	60-120	70-130†
Patient 1	8 bp deletion nts 1130-1139 & A5279G, Y1702C in trans	Compound heterozygous	< 1	< 3.1
Patient 2	1 bp deletion nts 4291/4294	Homozygous	< 1	< 3.1
Father		Heterozygous	57	56
Mother		Heterozygous	52	47
Patient 3	C2491T, Q773Term	Homozygous	< 1	ND
Patient 4	A1102T, K310Term	Heterozygous	34	51
Spouse		N	125	136
Child 1		Heterozygous	62	69
Child 2		Heterozygous	60	64
Child 3		N	109	142
Child 4		N	93	136
Patient 5 (III-1)	T1927C, C585R	Heterozygous	52	65
Father (II-4)		N	100	104
Mother (II-3)		Heterozygous	59	68
Sister (III-2)		Heterozygous	39	38
Uncle (II-2)		Heterozygous	55	59
Aunt (II-1)		Heterozygous	ND	66

N indicates normal; ND, not determined.
*As tested for the applicable mutation.
†Lower detection limit 3.1%

Table 2. FV gene amplification and sequencing primers

	Primer	Sequence (5′-3′)	Position	PCR product (bp)	Annealing (°C)
Promoter	HCVP-F	CTATGCTGCAGCTTAGCTGG	−684 to −665*	597	58
	HCVP-R	GCTGCAATGAGCTCTAGAGG	−88 to −107*		
Exon 1	HCFV-1F	AGGACGCTGCCACCCACAG	−126 to −108*	309	64
	HCFV-1R	CACCCGGACTCCACACCTG	intron 1, nt +25 to +7		
Exon 2	HCFV-2F	GTGAACAAATAGTTATCACAACAGG	intron 1, −223 to −199	383	59
	HCFV-2R	TGCATGTGAATGCCAAATTACC	intron 2, +68 to +47		
Exon 3	HCFV-3F	GATGACCCTGAATACAGACATAG	intron 2, −44 to −22	228	59
	HCFV-3R	GATGCTGGTATTAAAGACTTAGAC	intron 3, +61 to +38		
Exon 4	HCFV-4F	ACTGCCCACATGTCTTGATGG	intron 3, nt −36 to −16	311	58
	HCFV-4R	TGACAGAACTCCTGACCATTCC	intron 4, nt +62 to +41		
Exon 5	HCFV-5F	CTGCAGTGCTACTGAAAACATG	intron 4, nt −37 to −16	306	58
	HCFV-5R	TCCTTCTTGATAGGGAGTTGC	intron 5, nt +125 to +105		
Exon 6	HCFV-6F	GCCTAATCCTTTAGCAATCCCTG	intron 5, nt −290 to −268	547	58
	HCFV-6R	CATTGAGAAGCAAGACTGTCAGG	intron 6, nt +35 to +13		
Exon 7	HCFV-7F	GAGTTATTTCATTGTCTTTCTGTCC	intron 6, nt −33 to −9	241	58
	HCFV-7R	GTCTTGAACCTTTGCCCAG	intron 7, nt +42 to +24		
Exon 8	HCFV-8F	GCAGAATGTTTAAGCACAAGG	intron 7, nt −87 to −67	306	56
	HCFV-8R	CTATGTAATTTCTCCCATGATTCTG	intron 8, nt +41 to +17		
Exon 9	HCFV-9F	GATGACTTCAAAGACAGTGTCC	intron 8, nt −422 to −401	550	56
	HCFV-9R	GGATTCAGTAGAAGTGAAAGATTC	intron 9, nt +28 to +5		
Exon 10	HCFV-10F	ATGACAAGTTAATGGGTGCAGC	intron 9, nt −227 to −206	541	58
	FV-10R	CTTGAAGGAAATGCCCCATTA†	intron 10, nts +99 to +79		
Exon 11	HCFV-11F	TGGTCTATGCGTCTGTTCTTGTAC	intron 10, nt −50 to −27	250	62
	HCFV-11R	CAACCACAGGAATGAAAAACTG	intron 11, nt +49 to +28		
Exon 12	HCFV-12F	CATAGACTTGGAATTTTAACAG	intron 11, nt −37 to −16	286	50
	HCFV-12R	CAAGCTTCCTCTGTGAGTGTC	intron 12, nt +36 to +16		
Exon 13a	HCFV-13aF	TCCCAGACTTCCAGATCTCTC	intron 12, nt −44 to −24	605	60
	HCFV-13aR	CTGTGACATCTGGCTGTAGAGG	exon 13, nt 2626 to 2605		
Exon 13b	HCFV-13bF	CCAGCCCATATTCTGAAGACC	exon 13, nt 2573 to 2593	608	60
	HCFV-13bR	TCGTGTCTTAATGAGAAACTGGC	exon 13, nt 3180 to 3158		
Exon 13c	HCFV-13cF	TCTACAAGTAAGACAGGATGGAGG	exon 13, nt 3114 to 3137	626	60
	HCFV-13cR	GTTCTGGAGAGAGAGTCGTGTG	exon 13, nt 3739 to 3718		
Exon 13d	HCFV-L13F1	CAAGTCCTTCCCCACAGATATA‡	exon 13, nt 3579 to 3600	1002	59
	HCFV-L13R2	GTAGGAGATGAAGGAGATGG‡	exon 13, nt 4580 to 4561		
	HCFV-L13F2	ATGCCCCTCTTTGCAGATCT‡§	exon 13, nt 4261 to 4280		
	HCFV-L13R1	AGATCTGCAAAGAGGGGCAT‡§	exon 13, nt 4280 to 4261		
Exon 13e	HCFV-13eF	CTTCTGAATCTAGTCAGTCATTGC	exon 13, nt 4487 to 4510	450	60
	HCFV-13eR	TTCAGCAGTAATGGAAAAATGAG	intron 13, nt +50 to +28		
Exon 14	HCFV-14F	CTGACCTCATGGCACTTATACC	intron 13, nt −408 to −387	615	56
	HCFV-14R	CCGAAGATCTTAGCAGTGCTC	intron 14, nt +32 to +12		
Exon 15	HCFV-15F2	GGCCATATCTCACAGGATGG	intron 14, nt −177 to −158	600	56
	HCFV-15R	GTCATCTGAAGAGCTGCATGG	intron 15, nt +186 to +166		
Exon 16	HCFV-16F	AGTGCATGGTAAGCACTTGG	intron 15, nt −381 to −362	761	55
	HCFV-16R2	ACCTGCCAGATTACATCAGC	intron 16, nt +169 to +150		
Exon 17	HCFV-17F2	CCTTTCCATGGCTAGGTAGG	intron 16, nt −139 to −120	356	55
	HCFV-17R	TCTTAGCAGGGACCTCTTCC	intron 17, nt +37 to +18		
Exon 18	HCFV-18F	GAAAGCCTCTTGTGAAGCAGG	intron 17, nt −104 to −84	388	58
	HCFV-18R2	TTCAATGCAATCAGACCATGG	intron 18, nt +167 to +147		
Exon 19	HCFV-19F	ATTGAGTCAGAAACATAATCCC	intron 18, nt −95 to −74	222	58
	HCFV-19R	GCATGCTGCACAACTGTAGG	intron 19, nt +55 to +36		
Exon 20	HCFV-20F	AAGGATCTGGTTTTCCACTGG	intron 19, nt −93 to −73	366	57
	HCFV-20R2	ACCTCAGAGGGTTGATTTTAAGG	intron 20, nt +169 to +147		
Exon 21	HCFV-21F	GCAGTGTGTGACTTGTTGAC	intron 20, nt −57 to −38	241	58
	HCFV-21R	AGATTCAGATAGAAATATGCACAC	intron 21, nt +28 to +5		
Exon 22	HCFV-22F	TCTTCCTGGAACTGGAATTATCC	intron 21, nt −165 to −143	360	57
	HCFV-22R	TCTTGATTCTTTGAGTGGCAGTG	intron 22, nt +50 to +28		
Exon 23	HCFV-23F2	TGAGAACAGTATTTGGCACTTGG	intron 22, nt −162 to −140	398	58
	HCFV-23R	CCAGATCCTCCATGTTTGTGG	intron 23, nt +84 to +64		
Exon 24	HCFV-24F	AAGCAAAGGTTTTAACATCTTCC	intron 23, nt −52 to −30	258	56
	HCFV-24R	TCTTTGCCCAGATGCCAC	intron 24, nt +23 to +6		
Exon 25	HCFV-25F	CAGTCATACAGCTAATACAGACG	intron 24, nt −441 to −419	630	58
	HCFV-25R	GGTCTTAAAGAGTCTCTTCCAGG	exon 25, nt +42 to +20¶		

*Relative to the start codon (reference 14).
†From reference 63.
‡From reference 60.
§Additional primers used for DNA sequence analysis of exon 13d.
¶Relative to the termination codon (reference 14).

and DNA sequence analysis of the promoter and the individual exons are listed in Table 2. Intronic primers were chosen at appropriate distance from the splice sites. The large exon 13 was divided into 5 fragments (a-e). The PCR reactions were carried out with 50 to 100 ng DNA in 100 μL volumes containing 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM MgCl$_2$, 0.01% (wt/vol) gelatin, 0.2 mM of each dNTP, 0.3 μM of each primer, and 2.5 U AmpliTaq DNA polymerase. All reagents were obtained from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ). The samples were subjected to 30 or 35 cycles of amplification with denaturation at 94°C for 30 seconds (5 minutes at 95°C prior to the first cycle), annealing for 30 seconds at 50 to 64°C (Table 2) and extension at 72°C for 30 to 60 seconds followed by an elongated extension time of 10 minutes after the last cycle.

DNA sequence analysis

Automated DNA sequence analysis was performed with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Warrington, England), according to the manufacturer's instructions. Sequencing reactions were all carried out in forward and reverse direction, and samples were analyzed on an Applied Biosystems ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The PCR products were purified prior to DNA sequence analysis with the QIAquick PCR purification kit (Qiagen, Valencia, CA) or excised from the agarose gel and purified using the Prep-A-Gene DNA Purification Kit (Biorad Laboratories, Hercules, CA) according to instructions.

Reverse transcription-PCR

Reverse transcription-PCR (RT-PCR) to detect the T1927C mutation was performed using the GeneAmp RNA PCR Kit from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ) according to the instructions of the manufacturer. Briefly, 0.3 to 1.0 μg total RNA was reverse transcribed using random hexamers as primers. After addition of 30 pmol of primers HCFV-C11F 5′-ATGAGGTGAAACGTGATGACC-3′, exon 11 nts 1802 to 1822 and HCFV-C12R 5′-ACCGTCACAGATTCTCCACG-3′, exon 12 nts 2048 to 2029, the samples were subjected to 35 cycles of amplification with denaturation at 94°C for 30 seconds (5 minutes at 95°C prior to the first cycle), annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, followed by an elongated extension time of 10 minutes after the last cycle. Total liver RNA was used as a positive control and controls without RNA as well as controls in which the reverse transcription step was omitted were included.

Restriction enzyme analysis

All mutations were confirmed by restriction enzyme analysis on a newly amplified PCR product (Table 3). The 8-bp deletion in exon 7 was confirmed by *Mbo*II digestion. The PCR product as amplified with primers HCFV-7F and HCFV-7R is 241 bp and contains one restriction site for *Mbo*II. Digestion of the wild-type allele results in fragments of 134 and 107 bp. The deletion abolishes this site, rendering a single fragment of 233 bp.

To confirm the T1927C mutation, exon 12 was amplified with primers

Table 3. FV mutations and polymorphic frequencies detected in this study

Location	nt	Base change	Amino acid change	Restriction site*		Allele frequency	Reference
Promoter	−426	g → a	NA	*Bst*N I	+	0.86/0.14	This study
Exon 3	409	G → C	D79H	−		0.95/0.05	45
Exon 7	1102	A → T	K310Term; FV Amersfoort	−		ND	This study
Exon 7	1130-1139	8 bp deletion	frameshift; FV Seoul$_1$	*Mbo* II	+	ND	This study
Exon 12	1927	T → C	C585R; FV Nijkerk	*Bsl* I	−	1.00/0.00	This study
Exon 12	2016	C → A	T614	*Mnl* I	+	0.95/0.05	41, 45
Exon 13	2491	C → T	Q773Term; FV Casablanca	*Hph* I	+	ND	This study
Exon 13	2540	A → C	N789T	−		0.93/0.07	41, 45, 64
Exon 13	4185	C → T	T1337	−		0.61/0.39	This study
Exon 13	4279	C → T	L1369F	*Bst*Y I	+	0.99/0.01	41
Exon 13	4291-4294	1 bp deletion	frameshift; FV Utrecht	−		ND	This study
Exon 13	4300	C → T	P1376S	*Dde* I	−	0.99/0.01	41
Exon 15	5112	A → G	G1646	−		0.99/0.01	41, 45
Exon 15	5279	A → G	Y1702C; FV Seoul$_2$	*Cac*8 I	−	ND	25, this study
Exon 24	6533	T → C	M2120T	*Tai* I	−	0.99/0.01	This study
Exon 25	6755	A → G	D2194G	−		0.93/0.07	45, 62, 65
Intron 5	−262	t → c	NA	−		ND	This study
Intron 5	−195	g → a	NA	−		ND	This study
Intron 5	(−160)-(−157)	insertion t	NA	−		ND	This study
Intron 8	−387	g → a	NA	−		ND	This study
Intron 8	−374	g → t	NA	−		ND	This study
Intron 8	−121	t → c	NA	−		ND	This study
Intron 13	−323	t → c	NA	−		ND	This study
Intron 14	−88	c → t	NA	−		ND	This study
Intron 15	+73	g → a	NA	−		ND	This study
Intron 15	+365	a → t	NA	−		ND	This study
Intron 15	−325	t → g	NA	−		ND	This study
Intron 15	−134	t → c	NA	−		ND	This study
Intron 17	−178	a → g	NA	−		ND	This study
Intron 18	−50	g → a	NA	−		ND	This study
Intron 19	+349	c → g	NA	−		ND	This study
Intron 21	−44	t → c	NA	−		ND	This study
Intron 24	+130	a → c	NA	−		ND	This study
Intron 24	−147	g → a	NA	−		ND	This study
Intron 24	−65	a → c	NA	−		ND	This study

NA indicates not applicable; ND, not determined.

*Applicable restriction enzymes used for confirmation of mutations or polymorphisms and establishing allelic frequencies (n = 100). Presence (+) or absence (−) of a recognition site at this nucleotide position in the wild-type sequence is indicated.

HCFV-12F and HCFV-12R and subjected to *Bsl*I digestion. The 286-bp PCR product from the wild-type allele contains one restriction site for *Bsl*I, yielding fragments of 214 and 72 bp. The mutation creates a second restriction site resulting in additional fragments of 117 and 97 bp. The 247-bp PCR product containing exon 12, as obtained by RT-PCR with primers HCFV-C11F and HCFV-C12R, yields fragments of 228 and 19 bp for the wild-type allele and 131, 97, and 19 bp for the T1927C allele, following digestion with *Bsl*I.

The C2491T nonsense mutation in exon 13 was confirmed by *Hph*I digestion of the 5′ end of exon 13 as amplified with primers HCFV-13aF and HCFV-13aR. The 605-bp PCR product normally contains one recognition site for *Hph*I, producing fragments of 458 and 147 bp, which is abolished by this base change and thus results in an uncut PCR product from the mutant allele.

To detect the A5279G mutation, the PCR product (600 bp) amplified with primers HCFV-15F2 and HCFV-15R, containing exon 15, was subjected to digestion with *Cac*8I. The mutation adds an extra restriction site to the one normally present in this fragment; 338-, 55-, and 207-bp fragments are produced in contrast to the wild-type allele, 338 and 262 bp.

In general, 20 μL of the appropriate PCR product was incubated with 10 to 20 U of the respective enzymes. All enzymes were purchased from New England Biolabs (Beverly, MA).

Molecular modeling

A 3-dimensional diagram of the cysteine at residue 585 in FV was generated from an FV homology model[12] using computer programs Molscript[33] and Raster3D.[34]

## Results

FV activity and antigen

Factor V activity and antigen results of the propositae and relevant family members are summarized in Table 1. In all subjects, excluding patient 3 from whom plasma was unavailable, the reduction of FV activity correlated with a similar reduction of FV antigen, indicating a type I FV deficiency.

Molecular analysis

During the course of the DNA sequence analysis of the complete *FV* gene of the patients, relevant relatives, and a control subject, a number of novel base changes as compared to the published sequence were identified. One was located in the promoter, 15 in the coding region, and 19 in the noncoding region. The intronic base changes were excluded from being the (primary) genetic defect underlying the FV deficiency, based on the fact that they were not located in or near consensus sequence motifs considered critical for RNA processing, neither did they create any such sequence.[35,36] They were therefore considered to be polymorphisms. Likewise, the promoter base change at nt −426 was considered to be polymorphic given its high frequency in the control group. We report the allelic frequencies of all applicable exonic base changes, except for the 2 frameshift mutations and nonsense mutants, as determined in the control group. The results obtained from the molecular analysis are summarized in Table 3.

Patient 1

By DNA sequence analysis, this patient was found to be heterozygous for a novel deletion in exon 7, where 8 bp ((TG)AAGAG-G(TG)) were deleted between nts 1130 and 1139 (Figure 1), resulting in a frameshift and a premature stop codon at residue 351. This mutation was confirmed by *Mbo*II digestion. Because the patient had undetectable levels of FV activity and antigen (Table 1),
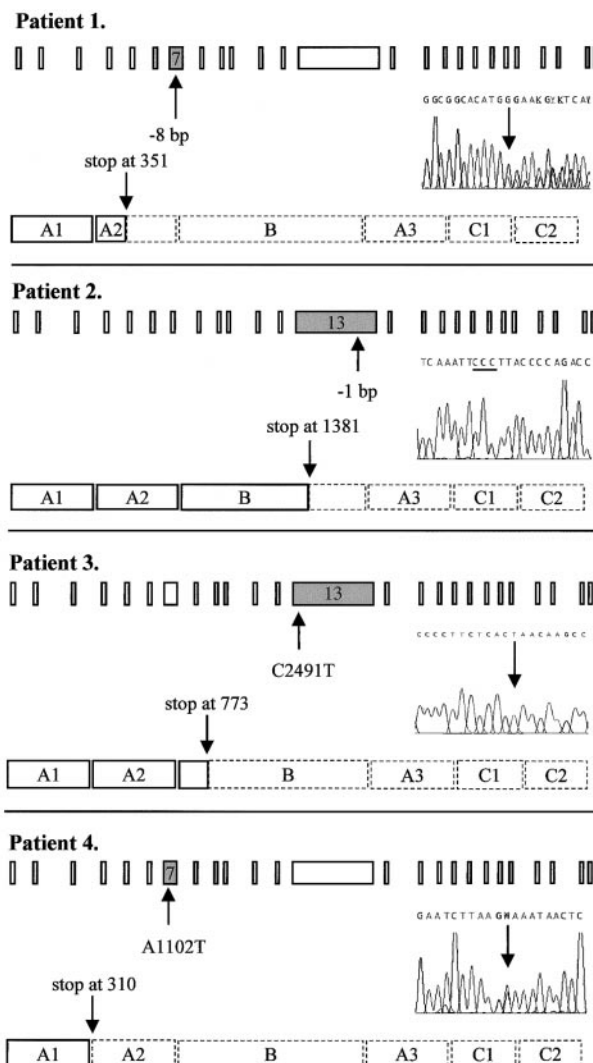
Figure 1. Predicted truncated FV molecules as encoded by the FV frameshift and nonsense mutations detected in this study. Schematic representation shows the 25 exons of the *FV* gene and the procoagulant protein that consists of a heavy chain (A1-A2), a connecting region or B domain, and a light chain (A3-C1-C2). The position of the mutation in the gene and in the DNA sequencing electropherogram as well as the predicted premature translation stop in the protein are indicated by arrows. The 3 cytosines resulting from the 1-bp deletion in patient 2 are underlined. Dashed lines in the protein indicate the part that is lacking, as compared to the wild-type protein. Patient 1: heterozygous 8-bp deletion between nts 1130 and 1139 in exon 7 leading to a premature stop at codon 351. Patient 2: homozygous 1-bp deletion between nts 4291 and 4294 in exon 13 leading to a premature stop at codon 1381. Patient 3: homozygous C→T nonsense mutation at nt 2491 in exon 13, changing the codon for glutamine at residue 773 into a stop codon. Patient 4: heterozygous A→T nonsense mutation at nt 1102 in exon 7, changing the codon for lysine at residue 310 into a stop codon. W: A or T

we postulated her to be a probable compound heterozygote. Further DNA sequence analysis revealed, apart from 6 previously published polymorphisms (G1628A,[37,38] (A2663G, A2684G, A2863G),[14,15,39,40] C4279T[41] and A5380G,[42-44]) one other base change in the heterozygous state: exon 15: A5279G, Y1702C. Because she was an orphan without any known relatives, it was not possible to determine which changes occurred *in trans* of the 8-bp deletion by linkage analysis. However, the A5279G mutation has recently been associated with FV deficiency[25] and is therefore most likely the causative mutation on the other FV allele of this patient. This missense mutation was confirmed by *Cac*8I digestion. We named this compound heterozygous variant FV Seoul$_{1,2}$.

Patient 2

DNA sequence analysis revealed a novel homozygous deletion in exon 13 where one cytosine was deleted from a series of 4 cytosines present between nts 4291 and 4294 (Figure 1). This resulted in a frameshift leading to a premature translation stop at codon 1381. Both parents, who were first cousins, were heterozygous for the same deletion thereby excluding the possibility of hemizygosity in the propositus due to a large deletion. We designated this variant FV Utrecht.

Patient 3

The *FV* gene of this patient showed a novel homozygous nonsense mutation in exon 13 where a C→T substitution at nt 2491 predicted a stop codon at residue 773 instead of the wild-type glutamine (Figure 1). This mutation was confirmed by *Hph*I digestion and we named this variant FV Casablanca.

Patient 4

This patient was found to have a single heterozygous A→T base change at nt 1102 in exon 7 (Figure 1), thereby changing the codon for lysine at residue 310 into a termination codon. All available family members were tested for this mutation and the nonsense mutation cosegregated with reduced FV activity and antigen levels (Table 1). This mutant was designated FV Amersfoort.

Apart from this nonsense mutation and a previously described heterozygous polymorphism (G409C),[45] we identified a novel homozygous base substitution in exon 24: T6533C, M2120T. Because this substitution concerns a conserved residue in the C2 domain of FV, its occurrence was investigated in the control group (Table 3). T6533C appeared to be a rare polymorphism of which all of the children in this family are obligate carriers, whereas the spouse of the patient is a wild-type homozygote (data not shown). The 2 children in this family who lack the A1102T mutation had normal FV activity levels (Table 1), so any (additional) effect of this amino acid substitution to the more pronounced reduction in FV activity of the propositus compared to his 2 sons (34% versus 62% and 60%) was not likely to be due to the fact that he was homozygous for this rare polymorphism.

Patient 5

We identified 6 nucleotide substitutions linked to lowered activity and antigen levels in this family. Five were previously reported polymorphisms: (A2663G, A2684G, A2863G),[14,15,39,40] C4300T,[41] and A5380G,[42-44] whereas one was a novel T→C substitution at nt 1927 in exon 12, coding for a C→R change at residue 585. We investigated the occurrence of T1927C in a normal population but found no control subjects carrying this allele (Table 3). We postulated the T1927C base change to cause FV deficiency (see "Discussion") in this family (Table 1 and Figure 2) and named this variant FV Nijkerk. The mutant allele was transcribed to RNA, because the mutant RNA could be detected, by RT-PCR, in both the patient and his mother (Figure 2).

Discussion

Little is known about the molecular basis underlying FV deficiency due to its low frequency in the population combined with the complexity of the gene itself. In this study we investigated 3 patients with severe FV deficiency and 2 asymptomatic carriers
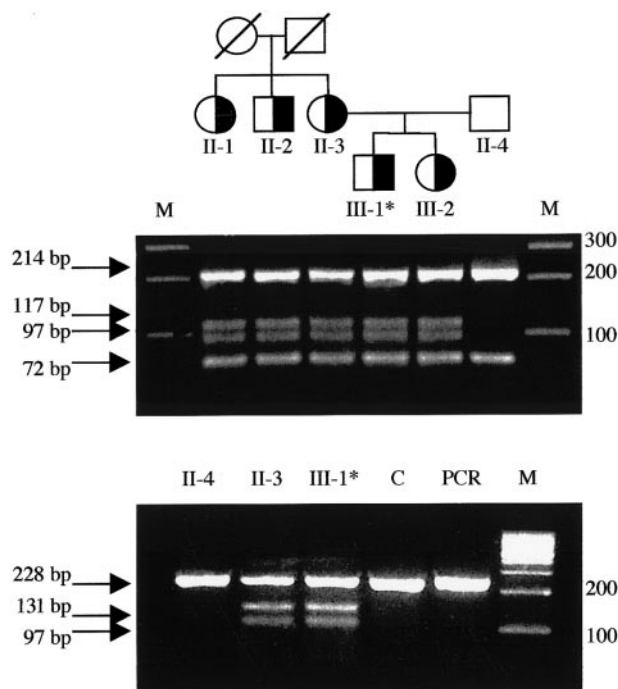
Figure 2. Detection of the T1927C missense mutation in the family pedigree and mutant RNA. (Top) Family pedigree: individuals heterozygous for the T1927C (FV Nijkerk) missense mutation are indicated by a half-closed symbol. (Middle) Exon 12 was amplified from genomic DNA and subjected to *Bsl* I digestion as described. The mutation creates an additional restriction site and the resulting fragments are indicated by arrows. Each lane represents the DNA from the individuals located directly above in the family pedigree in the top panel. (Bottom) Total RNA was reverse transcribed and amplified as described. Subsequent *Bsl* I digestion revealed the presence of both the wild-type allele as well as the mutant allele in the RNA of patient 5 (III-1) and his mother (II-3). Asterisk indicates propositus; M, marker; C, liver tissue[32] RNA control; PCR, uncut RT-PCR product (247 bp).

from 5 different families. We identified 5 novel mutations and a recently published mutation associated with FV deficiency.

Patients 1, 2, and 3 all had severe FV deficiency and undetectable levels of FV. DNA sequence analysis of the *FV* gene revealed 2 novel frameshift mutations and a novel nonsense mutation associated with an FV null allele. Patients 2 and 3 were both homozygous for a mutation in exon 13, respectively, a 1-bp deletion between nts 4291 and 4294 and a C2491T nonsense mutation. Patient 1 was compound heterozygous for an 8-bp deletion in exon 7 and a missense mutation in exon 15: A5279G, Y1702C (FV Seoul₂). The missense mutation was recently described in the heterozygous state as causative for FV deficiency.[25] Our study provides further evidence for the postulation that the Y1702C change leads to FV deficiency, because neither FV activity nor FV antigen could be detected in our patient.

The 2 small deletions and the nonsense mutation in the *FV* genes of patients 1, 2, and 3 would, if translated, result in truncated FV molecules. The 8-bp deletion between nts 1130 and 1139 in exon 7, −(TG)AAGAGG(TG) (FV Seoul₁), in patient 1 results in a frameshift and would code for a peptide of only 350 amino acids long, lacking approximately half the heavy chain and the entire B domain and light chain (Figure 1). In patient 2, the deletion of a cytosine between nts 4291 and 4294 in exon 13 (FV Utrecht) results in a frameshift that would code for a premature translation stop at codon 1381. Consequently, this protein would lack approximately 20% of the B domain and the entire light chain (Figure 1). In patient 3, the C2491T nonsense mutation in exon 13 (FV Casablanca) predicts a premature stop instead of a glutamine at residue

773 and thereby encodes a protein that would consist of a FV heavy chain and only 63 amino acids of the B domain (Figure 1). Thus, this variant FV would lack approximately 90% of the B domain and the complete light chain.

In general, nonsense mRNAs resulting from frameshift and nonsense mutations are highly unstable, because they are subjected to nonsense-mediated mRNA decay (NMD),[46-48] preventing the potentially deleterious effects of truncated proteins. Recent studies of FV RNA metabolism demonstrated the absence of RNA of the *in trans* non-FV Leiden allele in patients who are "pseudo homozygous" for FV Leiden due to either a nonsense mutation[21] or a frameshift mutation[19] in exon 13. Consequently, the frameshift and nonsense mutations in patients 1, 2, and 3 most likely are associated with an FV null allele. The patients presented with different phenotypes: patients 1 and 2 had moderate bleeding symptoms, whereas patient 3 was asymptomatic. Furthermore, patient 1 suffered from spontaneous muscle bleedings, whereas these were absent in patient 2. This variability in phenotypic expression has previously been observed in other FV-deficient patients. Moreover, as mentioned in earlier reports,[49,50] there is a striking discrepancy between mice and man. FV null mice die either in utero or of fatal perinatal hemorrhage,[49] whereas human patients with undetectable FV levels do survive. Table 4 summarizes the clinical phenotype of all patients with severe FV deficiency described to date who have been characterized at the molecular level. There is a marked difference in age at diagnosis and clinical presentation. As shown by patients I, VI, XI, and XII in Table 4, similar residual amounts of FV are still associated with a different phenotypic expression. Also, individuals with the same mutation, between families (patients I and VI) and even within families (patients II and VII), show a different clinical bleeding pattern. Therefore, the clinical expression of FV deficiency most likely does not depend solely on the type of mutation but also on other, yet unknown, modulating factors.

Previously it has been proposed[50,51] that in patients with severe FV deficiency a very low level of expression of FV may occur. Because trace amounts of FV are capable of generating a significant amount of thrombin,[52] this would then result in a

level of FV synthesis sufficient to mitigate the clinical phenotype. For instance, "ribosomal frameshifting" has previously been recognized as a mechanism able to partially rescue protein synthesis from genes harboring frameshift mutations,[53-55] and it has also been proposed to play a role in the phenotypic expression of FV deficiency caused by a frameshift mutation in exon 13.[50] Mutations in exon 13 occur frequently in the *FV* gene (Table 5) and because the B domain is relatively unimportant for FV procoagulant function, "ribosomal frameshifting" may thereby reflect one of the modulating factors involved in the phenotypic differences observed in these patients. As shown in Table 4, however, mutations in exon 13 still can lead to a severe phenotypic expression of the disease.

In mammals, the basic mechanism by which nonsense transcripts are recognized and subjected to NMD is still poorly understood.[48] Therefore, it cannot be excluded that any residual amount of stable nonsense FV mRNA could actually produce small amounts of (truncated) protein. These molecules could escape detection by the methods used, but still be of interest for their remaining function or potentially harmful effects in the procoagulant and anticoagulant pathways. In humans, the role of NMD as a modifier of the phenotypic consequences of nonsense mutations is becoming increasingly evident, for example, in β-thalassemia.[56-58] Clearly, multiple factors are involved in modulating the phenotypic expression of FV deficiency. Therefore, studies using genotypic and phenotypic data are warranted to obtain insight in the mechanisms leading to the observed differences in phenotypic expression of severe FV deficiency.

Patients 4 and 5 were both asymptomatic and carriers of a novel FV null allele. However, because the presence and function of these FV variants could be studied only in individuals heterozygous for the mutation, we cannot exclude the presence of small amounts of mutant protein that would go by undetected because of the wild-type background. In patient 4, a novel nonsense mutation was identified: A1102T, K310Term (FV Amersfoort) that would code for a truncated protein lacking the A2 domain of the heavy chain, the complete B domain and the entire light chain (Figure 1). This mutation was found to cosegregate within the affected family with

Table 4. Phenotypic expression of FV deficiency

Patient*	Phenotype	Mutation	Exon	Mutation type	Genotype	Gender	Age at diagnosis	FV activity	FV antigen	Reference
I	Asymptomatic	C3571T	13	Nonsense	Homozygous	Male	7 y	< 1%	0.2%	†
II	Asymptomatic	C2491T	13	Nonsense	Homozygous	Female	2 y	< 1%	ND	This study
III	Very mild	−4 bp nts 4014/7	13	Frameshift	Homozygous	Female	3 y	< 1.6%	< 0.3%	20
IV	Mild	G6395A	23	Missense	Homozygous	Male	NR	NR	< 5%	29
V	Mild	G6395A	23	Missense	Homozygous	Female	NR	NR	< 5%	29
VI	Mild	C3571T	13	Nonsense	Homozygous	Female	10 y	< 1%	0.2%	†
VII	Mild	C2491T‡	13	Nonsense	Homozygous	Male	10 y	< 3%	NR	31
VIII	Moderate	−1 bp nts 4291/4	13	Frameshift	Homozygous	Male	1 yr	< 1%	< 3.1%	This study
IX	Moderate	−8 bp exon 7 & A5279G	7 & 15	Frameshift & missense	Compound heterozygous	Female	1 y	< 1%	< 3.1%	This study
X	Severe	−2 bp nts 2833/4	13	Frameshift	Homozygous	Male	2 y	< 1%	< 0.2%	30
XI	Severe	−1 bp nts 2857/9	13	Frameshift	Homozygous	Female	Birth	NR	0.02 IU/mL	26
XII	Severe	−1 bp nts 2857/9	13	Frameshift	Homozygous	Female	Birth	NR	0.02 IU/mL	26

Patient III: posttraumatic oral cavity bleeding, easy bruising; patients IV and V: no further information; patient VI: oral cavity bleeding, posttraumatic thigh hematoma, menorrhagia; patient VII: prolonged bleeding after injuries; patient VIII: oral cavity bleeding, epistaxis, hematomas; patient IX: oral cavity bleeding, epistaxis, hematomas, spontaneous muscle bleedings; patient X: gastrointestinal bleeding, epistaxis, knee and ankle hemarthrosis, hematuria, iliopsoas hematoma; patient XI: hydrocephalus, menorrhagia, intra-abdominal hemorrhage, follicular bleeding; patient XII: cephal-hematoma, (knee and ankle) hemarthrosis, subdural hematoma.

ND indicates not determined; NR, not reported.

*Patients II, VIII, and IX correspond with this study's patients 3, 2, and 1, respectively.

†van Wijk R and coworkers, manuscript submitted.

‡Brother of patient 3. His DNA was not available to confirm homozygosity for the C2491T mutation.

Table 5. Mutations in the Factor V gene associated with FV deficiency

Nucleotide change	Exon	Effect	Name	Reference
I. Nonfunctional mRNA				
Nonsense mutants				
1. A1102T	7	K310Term	FV Amersfoort	This study
2. C1690T	10	R506Term		22, 23
3. C2308T	13	R712Term		21
4. C2491T	13	Q773Term	FV Casablanca	This study
5. C3571T	13	R1133Term		28
Frameshift mutants				
1. 8 bp deletion nts 1130-1139 −(TG)AAGAGG(TG)	7	Frameshift	FV Seoul₁	This study
2. 2 bp deletion nts 2833-2834 −AC	13	Frameshift		30
3. 1 bp deletion nts 2857-2859 −C	13	Frameshift		26
4. 2 bp insertion nt 3706 +TC	13	Frameshift		19
5. 4 bp deletion nts 4014-4017 −TCAG	13	Frameshift		20
6. 1 bp deletion nts 4291-4294 −C	13	Frameshift	FV Utrecht	This study
7. 4 bp insertion nt 4793 +ATTG	13	Frameshift	FV Stanford	24, 27
II. RNA processing				
Consensus changes				
1. G5509A	16	Splicing		18
III. Coding region substitutions				
Missense mutants				
1. C836T	6	A221V	FV New Brunswick	17
2. T1927C	12	C585R	FV Nijkerk	This study
3. A5279G	15	Y1702C	FV Seoul₂	25, this study
4. G6395A	23	R2074H		29

decreased FV activity and antigen levels. In patient 5, a novel base change was detected: T1927C, C585R (FV Nijkerk) that most likely represents the mutation associated with the decrease in FV activity and antigen levels. This missense mutation cosegregated with FV deficiency in the affected family and was not detected among 100 normal alleles. Although mutant RNA was detected, indicating transcription from the FV Nijkerk allele, FV antigen and activity levels were consistent with heterozygous type I FV deficiency. Apparently, substitution of C585 by arginine results in rapid degradation of FV, either before or immediately after secretion into the circulation. C585 is conserved among human, bovine, and murine FV, but not in FVIII and ceruloplasmin. Analysis of disulfide bridges and free cysteines in bovine FVa have not yielded conclusive data on the state of C589 (C585 in human FV).[59] However, homology modeling of FV A domains based on the crystal structure of ceruloplasmin indicates that C585 is not involved in disulfide-bond formation.[12] In the FV homology model C585 is completely buried inside the hydrophobic interior of the A2 domain. Substitution of a small and hydrophobic cysteine by a large and hydrophilic arginine would disrupt the tight packing interactions typical of protein interiors. As a consequence, the protein might be unstable and prone to intracellular proteolysis. If stable FV Nijkerk would be synthesized, functionality is doubtful because a potential thrombin cleavage site is created by the introduction of an arginine at residue 585, adjacent to S586. Cleavage by thrombin at this site would result in excision of a fragment from amino acids 585 to the next thrombin cleavage site at R709. This would disrupt the C-terminal end of the A2 domain, resulting in a loss of function or unstable protein.

Noteworthy with respect to the sister of the propositus in the family carrying the FV Nijkerk variant is the fact that her FV levels were consistently lower than in the other carriers of the T1927C mutation (Table 1). This may be explained by the fact that she inherited a FV HR2 allele from her father (data not

shown). This variant FV, characterized by the H1299R substitution in the FV B domain, has been associated with reduced FV levels.[60-62] Phenotypically, she is therefore "pseudo homozygous" for FV HR2, a rare FV genotype that has been reported previously once by Castoldi and coworkers.[25] They reported a family in which one member carried a FV missense mutation (Y1702C), associated with absence of the mutant protein in plasma, on the allele *in trans* of the FV HR2 allele. This family member also had lower FV levels as compared to carriers of only the missense mutation in the family.[25]

This study brings the total number of mutations associated with FV deficiency described to date to 17. An overview is presented in Table 5 where the mutations are listed according to their proposed mode of action. The majority of these mutations have been found in unique families; only the C1690T,[22,23] C3571T,[28] A5279G (this study and reference 25) and G6395A[29] have been found in more than one (unrelated) family. FV Seoul₁,₂ in this study is the first compound heterozygous patient described to date who is fully characterized at the molecular level and in whom 2 rare FV deficiency–associated mutations are combined that completely abolish FV synthesis. Identifying the molecular basis underlying this rare coagulation disorder will help to obtain more insight into the mechanisms involved in the variable clinical phenotype of FV-deficient patients.

References

1. Jenny RJ, Tracy PB, Mann KG. The physiology and biochemistry of factor V. In: Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD, eds. Haemostasis and Thrombosis. 3rd ed. Edinburgh: Churchill Livingstone; 1994:465-476.

2. Tuddenham EGD, Cooper DN. Factor V: the Molecular Genetics of Haemostasis and Its Inherited Disorders. Oxford Monographs on Medical Genetics. Oxford, United Kingdom: Oxford University Press; 1994:182-193.

3. Rosing J, Tans G. Coagulation factor V: an old star shines again. Thromb Haemost. 1997;78:427-433.

4. Hoekema L, Nicolaes GA, Hemker HC, Tans G, Rosing J. Human factor Va$_1$ and factor Va$_2$: properties in the procoagulant and anticoagulant pathways. Biochemistry. 1997;36:3331-3335.

5. Nicolaes GA, Villoutreix BO, Dahlbäck B. Partial glycosylation of Asn2181 in human factor V as a cause of molecular and functional heterogeneity: modulation of glycosylation efficiency by mutagenesis of the consensus sequence for N-linked glycosylation. Biochemistry. 1999;38:13584-13591.

6. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem. 1994;269:31869-31880.

7. Váradi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor V R506Q mutation. Thromb Haemost. 1996;76:208-214.

8. Church WR, Jernigan RL, Toole J, et al. Coagulation factors V and VIII and ceruloplasmin constitute a family of structurally related proteins. Proc Natl Acad Sci U S A. 1984;81:6934-6937.

9. Kane WH, Davie EW. Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood. 1988;71:539-555.

10. Macedo-Ribeiro S, Bode W, Huber R, et al. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. Nature. 1999;402:434-439.

11. Villoutreix BO, Bucher P, Hofmann K, Baumgartner S, Dahlbäck B. Molecular models for the two discoidin domains of human blood coagulation factor V. J Mol Model. 1998;4:268-275.

12. Villoutreix BO, Dahlbäck B. Structural investigation of the A domains of human blood coagulation factor V by molecular modeling. Protein Sci. 1998;7:1317-1325.

13. Koeleman BP, Reitsma PH, Bakker E, Bertina RM. Location on the human genetic linkage map of 26 genes involved in blood coagulation. Thromb Haemost. 1997;77:873-878.

14. Jenny RJ, Pittman DD, Toole JJ, et al. Complete cDNA and derived amino acid sequence of human factor V. Proc Natl Acad Sci U S A. 1987;84:4846-4850.

15. Cripe LD, Moore KD, Kane WH. Structure of the gene for human coagulation factor V. Biochemistry. 1992;31:3777-3785.

16. Owren PA. Parahaemophilia: haemorrhagic diathesis due to absence of a previously unknown clotting factor. Lancet. 1947:446-448.

17. Murray JM, Rand MD, Egan JO, Murphy S, Kim HC, Mann KG. Factor V$_{New\ Brunswick}$: Ala221-to-Val substitution results in reduced cofactor activity. Blood. 1995;86:1820-1827.

18. Guasch JF, Lensen RP, Bertina RM. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is "pseudo homozygous" for activated protein C resistance. Thromb Haemost. 1997;77:252-257.

19. Castoldi E, Kalafatis M, Lunghi B, et al. Molecular bases of pseudo-homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. Thromb Haemost. 1998;80:403-406.

20. Guasch JF, Cannegieter S, Reitsma PH, van 't Veer-Korthof ET, Bertina RM. Severe coagulation factor V deficiency caused by a 4 bp deletion in the factor V gene. Br J Haematol. 1998;101:32-39.

21. Lunghi B, Castoldi E, Mingozzi F, Bernardi F, Castaman G. A novel factor V null mutation detected in a thrombophilic patient with pseudo-homozygous APC resistance and in an asymptomatic unrelated subject [letter]. Blood. 1998;92:1463-1464.

22. Kitzis A, Salmeron S, Chomel JC, Boinot C, Patri S. Hum Genet. Gene symbol: FV; disease: factor V deficiency. 1999;104:290.

23. Mirochnik O, Halim-Kertanegara N, Henniker AJ, et al. A novel factor V null mutation at Arg 506 causes a false positive Factor V Leiden result [letter]. Thromb Haemost. 1999;82:1198-1199.

24. Zehnder JL, Hiraki DD, Jones CD, Gross N, Grumet FC. Familial coagulation factor V deficiency caused by a novel 4 base pair insertion in the factor V gene: factor V Stanford. Thromb Haemost. 1999;82:1097-1099.

25. Castoldi E, Simioni P, Kalafatis M, et al. Combinations of 4 mutations (FV R506Q, FV H1299R, FV Y1702C, PT 20210G/A) affecting the prothrombinase complex in a thrombophilic family. Blood. 2000;96:1443-1448.

26. Howard J, Hughes RG, Basu S, et al. A novel mutation in the factor V gene causing a severe coagulation defect in two sisters [abstract]. Blood. 2000;96:642a.

27. Montefusco MC, Asselta R, van Wijk R, Duga S, Tenchini ML. Concerns about the mutations identified in a case of familial coagulation factor V deficiency: factor V Stanford [letter]. Thromb Haemost. 2000;84:1131-1132.

28. Montefusco MC, Duga S, Asselta R, et al. Severe factor V deficiency caused by two novel mutations in exon 13 of factor V gene [abstract]. Haematologica. 2000;85(suppl to n. 5):95.

29. Schrijver I, Houissa-Kastally R, Jones CD, Zehnder JL. Identification of a novel factor V C2-domain mutation (R2074H) in two separate families with factor V deficiency and bleeding [abstract]. Blood. 2000;96:533a.

30. Montefusco MC, Duga S, Asselta R, et al. A novel 2 bp deletion in the factor V gene associated with severe factor V deficiency. Br J Haematol. 2000;111:1240-1246.

31. Tanis BC, van der Meer FJ, Bloem RM, Vlasveld LT. Successful excision of a pseudotumour in a congenitally factor V deficient patient. Br J Haematol. 1998;100:380-382.

32. Lind B, van Solinge WW, Schwartz M, Thorsen S. Splice site mutation in the human protein C gene associated with venous thrombosis: demonstration of exon skipping by ectopic transcript analysis. Blood. 1993;82:2423-2432.

33. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Cryst. 1991;24:946-950.

34. Merritt EA, Bacon DJ. Raster3D: photorealistic molecular graphics. Methods Enzymol. 1997;277:505-524.

35. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet. 1992;90:41-54.

36. Cooper DN, Krawczak M, Antonarakis SE. The nature and mechanisms of human gene mutation. In: Scriver C, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular basis of inherited disease. 7th ed. New York, NY: McGraw Hill; 1995:259-291.

37. Gandrille S, Greengard JS, Alhenc-Gelas M, et al. Incidence of activated protein C resistance caused by the ARG 506 GLN mutation in factor V in 113 unrelated symptomatic protein C-deficient patients. The French Network on the behalf of INSERM. Blood. 1995;86:219-224.

38. Helley D, Besmond C, Ducrocq R, et al. Polymorphism in exon 10 of the human coagulation factor V gene in a population at risk for sickle cell disease. Hum Genet. 1997;100:245-248.

39. Kane WH, Ichinose A, Hagen FS, Davie EW. Cloning of cDNAs coding for the heavy chain region and connecting region of human factor V, a blood coagulation factor with four types of internal repeats. Biochemistry. 1987;26:6508-6514.

40. Shen NL, Fan ST, Pyati J, Graff R, LaPolla RJ, Edgington TS. The serine protease cofactor factor V is synthesized by lymphocytes. J Immunol. 1993;150:2992-3001.

41. Ohnishi Y, Tanaka T, Yamada R, et al. Identification of 187 single nucleotide polymorphisms (SNPs) among 41 candidate genes for ischemic heart disease in the Japanese population. Hum Genet. 2000;106:288-292.

42. Kane WH, Davie EW. Cloning of a cDNA coding for human factor V, a blood coagulation factor homologous to factor VIII and ceruloplasmin. Proc Natl Acad Sci U S A. 1986;83:6800-6804.

43. Bayston TA, Ireland H, Olds RJ, Thein SL, Lane DA. A polymorphism in the human coagulation factor V gene. Hum Mol Genet. 1994;3:2085.

44. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va [letter]. Lancet. 1994;343:1361-1362.

45. Cargill M, Altshuler D, Ireland J, et al. Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nat Genet. 1999;22:231-238.

46. Culbertson MR. RNA surveillance: unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet. 1999;15:74-80.

47. Czaplinski K, Ruiz-Echevarría MJ, González CI, Peltz SW. Should we kill the messenger? The role of the surveillance complex in translation termination and mRNA turnover. Bioessays. 1999;21:685-696.

48. Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet. 1999;8:1893-1900.

49. Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature. 1996;384:66-68.

50. Yang TL, Cui J, Taylor JM, Yang A, Gruber SB, Ginsburg D. Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost. 2000;83:70-77.

51. Lak M, Sharifian R, Peyvandi F, Mannucci PM. Symptoms of inherited factor V deficiency in 35 Iranian patients. Br J Haematol. 1998;103:1067-1069.

52. Mann KG. How much factor V is enough? Thromb Haemost. 2000;83:3-4.

53. Hu PY, Waheed A, Sly WS. Partial rescue of human carbonic anhydrase II frameshift mutation by ribosomal frameshift. Proc Natl Acad Sci U S A. 1995;92:2136-2140.

54. Linton MF, Raabe M, Pierotti V, Young SG. Reading-frame restoration by transcriptional slippage at long stretches of adenine residues in mammalian cells. J Biol Chem. 1997;272:14127-14132.

55. Young M, Inaba H, Hoyer LW, Higuchi M, Kazazian HH Jr, Antonarakis SE. Partial correction of a

severe molecular defect in hemophilia A, because of errors during expression of the factor VIII gene. Am J Hum Genet. 1997;60:565-573.

56. Hall GW, Thein S. Nonsense codon mutations in the terminal exon of the β-globin gene are not associated with a reduction in β-mRNA accumulation: a mechanism for the phenotype of dominant β-thalassemia. Blood. 1994;83:2031-2037.

57. Thermann R, Neu-Yilik G, Deters A, et al. Binary specification of nonsense codons by splicing and cytoplasmic translation. EMBO J. 1998;17:3484-3494.

58. Romão L, Inácio A, Santos S, et al. Nonsense mutations in the human beta-globin gene lead to unexpected levels of cytoplasmic mRNA accumulation. Blood. 2000;96:2895-2901.

59. Xue J, Kalafatis M, Silveira JR, Kung C, Mann KG. Determination of the disulfide bridges in factor Va heavy chain. Biochemistry. 1994;33:13109-13116.

60. Lunghi B, Iacoviello L, Gemmati D, et al. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. Thromb Haemost. 1996;75:45-48.

61. Alhenc-Gelas M, Nicaud V, Gandrille S, et al. The factor V gene A4070G mutation and the risk of venous thrombosis. Thromb Haemost. 1999;81:193-197.

62. de Visser MC, Guasch JF, Kamphuisen PW, Vos HL, Rosendaal FR, Bertina RM. The HR2 haplotype of factor V: effects on factor V levels, normalized activated protein C sensitivity ratios and the

risk of venous thrombosis. Thromb Haemost. 2000;83:577-582.

63. Koeleman BP, Reitsma PH, Allaart CF, Bertina RM. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. Blood. 1994;84:1031-1035.

64. Kostka H, Siegert G, Gehrisch S, Kuhlisch E, Runge E, Jaross W. New missense mutation in exon 13 (N789T) of the coagulation factor V gene. Hum Mutat. 1999;14:357.

65. Castoldi E, Rosing J, Girelli D, et al. Mutations in the R2 FV gene affect the ratio between the two FV isoforms in plasma. Thromb Haemost. 2000;83:362-365.