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New mutations in exon 28 of the von Willebrand factor gene detected in patients with different types of von Willebrand's disease

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Background and Objectives. von Willebrand's disease (vWD), the most common hereditary bleeding disorder in humans, is caused by qualitative and/or quantitative deficiencies of von Willebrand factor, and can manifest itself under several different phenotypes. Most of the molecular defects have been detected in qualitative variants involving exon 28 of the vWF gene. Patients from four unrelated families with different types of vWD were included in the mutation screening of this region.

Design and Methods. The whole exon 28 was analyzed in three gene specific fragments, two of them comprising the region involved in the platelet glycoprotein Ib vWF interaction. The search for mutations was carried out by single-stranded conformation polymorphism analysis. The mutations were then identified by automatic sequencing of the anomalous electrophoretic pattern samples.

Results. The following candidate mutations were detected. The 3941T→A transversion, which predicts the amino acid change V1314D, was detected in a sporadic patient with type 2B vWD and severe thrombocytopenia. The 4309G→A transition, resulting in the amino acid substitution A1437T, was identified in four patients classified as having type 2M vWD. Six unclassified patients from another family carry the 4135C→T mutation, which gives rise to a cysteine instead of the normal arginine (R1379C) that segregates with the phenotype. The amino acid change C1227R, predicted by the mutation 4135C→T, was identified as a compound heterozygote in a patient with moderately severe type 1 vWD. None of these mutations had been described previously.

Interpretation and Conclusions. These findings confirm the importance already given to this region for the correct function of von Willebrand factor since the mutations detected, which affect the D3 and A1 domains, could give rise to different variants of the disease.

Key words: von Willebrand's disease, type 2B vWD, type 2M vWD, type 1 vWD, mutation detection

Von Willebrand's disease (vWD), the most common hereditary bleeding disorder, is caused by qualitative and/or quantitative abnormalities of von Willebrand factor (vWF). The vWF gene, located on the short arm of chromosome 12, spans 178 Kb in length and contains 52 exons.[1] On chromosome 22 there is a partial pseudogene with about 97% homology with exons 23-34 of the vWF gene, which makes analyses of this latter region difficult.[2] The revised classification[3] of vWD distinguishes three types of vWF deficiencies: partial quantitative (type 1), qualitative (type 2), and total quantitative (type 3). Qualitative defects are broken down into four subcategories (2A, 2B, 2M, and 2N). Most of the molecular defects have been detected in qualitative variants because the different phenotypes are confined to specific regions of the vWF gene. Nearly 200 mutations have been registered in the vWF database (*http://mmg2.im.med.umich.edu/vWF/*). Approximately 72% of them are recorded as qualitative variants. The majority of mutations causing types 2A, 2B, and 2M are confined to exon 28. Mutations in quantitative variants, by contrast, may lie on the whole vWF gene, making mutation detection a costly and painstaking process. Approximately 18% of genetic defects registered in the database correspond to type 3 vWD and 4% to type 1 vWD, while 6% remain unclassified. This study reports on the characterization of new missense mutations in the D3 and A1 domains, which are involved in the platelet glycoprotein Ib (GPIb) vWF interaction, associated with different variants of vWD.

Design and Methods

Patients

The propositus from family #1 is a seven-year old girl who had a first bleeding symptom at the age of three months, following her first vaccination. She has severe thrombocytopenia (9,600-80,000 platelets/μL). At first, immune thrombocytopenic purpura (ITP) was diagnosed, for which she was treated with γ-globulins and corticosteroids. However, as the platelet count and the hemorrhagic diathesis were not corrected, it was pro-

posed that she undergo splenectomy. The pre-operation tests revealed a prolongation of the activate partial thromboplastin time. Hemostatic studies showed a decrease of vWF levels with very low functional values, absence of high molecular weight multimers, and decrease of medium molecular weight multimers. A high affinity of plasma vWF for ristocetin-dependent platelet receptor was confirmed, and the patient was re-diagnosed as having type 2B vWD.

The four patients from family #2, who have moderate-mild bleeding symptoms, were classified as having type 2M vWD on account of the disagreement between functional and antigenic vWF levels, which showed a ratio of about 0.5 in several determinations.

The six patients from family #3 showed mild bleeding symptoms and were diagnosed as having type I vWD, according to the old classification.

The *propositus* from family #4, previously diagnosed as having type I vWD, has a history of moderate to severe bleeding. He was classified as having type 1/2 on the basis of the slight increase of agglutination observed in ristocetin-induced platelet agglutination (RIPA) analysis, performed on both platelet-rich and platelet-poor plasma samples (Table 1). There is mention of a history of mild bleeding in the maternal branch of the family, but not in the paternal branch.

The patients' relatives were also studied. The families gave their consent after they were informed of the kind of study they were taking part in. The functional, antigenic and multimeric assays were carried out as described previously.[4] A summary of the results from the patients are shown in Table 1.

Genetic analyses

DNA was extracted from blood collected in EDTA using a standard method. For indirect analysis, the following microsatellites were commonly analyzed: VNTR3, VNTR1 and VNTR2 (all of them located in intron 40 of the vWF gene, corresponding to nucleotides 1640-1793, 1890-1991, and 2215-2380, respectively),[5] and another in the promoter region (VWP, nucleotides 1490-1665). The *RsaI* and *HphI* polymorphisms located on exon 18 (15/292 nucleotide) and exon 28 (24/672) were also routinely studied. The nucleotide numbering used is that described by Mancuso *et al*.[1] The whole exon 28 was analyzed in three specific fragments, two of which comprise most of the region involved in the platelet GPIb vWF interaction. The search for mutations was carried out by single-strained conformation polymorphism analysis (SSCP). The 5'-end of the exon 28 was analyzed as previously described,[4] and comprises a fragment of 487 bp extending from intron 27 to codon 1368. The second fragment of 294 bp includes the 8001-8294 nucleotides.[2] The amplifications of the genomic DNA from both fragments were specific for the gene as checked by restriction analysis with *DdeI* and *TaqI*, respectively. The third fragment, spanning 770 bp of the 3' end of the exon 28, was also studied and was gene specific as checked by NcoI nucleotide 8229-8998.[2] The amplified DNA was purified

Table 1. Laboratory analytical data.

	BG	vWF:RCo (U/dL)	vWF:Ag (U/dL)	FVIII:C (U/dL)	RIPA PRP (mg/mL)	RIPA PPP (mg/mL)
Family 1						
II:1[a]		7	28	36	-	0.4
Family 2						
I:2		17 ± 11	33 ± 4	56 ± 17	1.2	-
II:1		14 ± 4	30 ± 7	69 ± 10	1.2	-
II:2		18 ± 3	42 ± 9	73 ± 24	1.2	-
II:4		14	29	86	-	-
Family 3						
II:1	O^+	18	29	52	-	-
II:3	O^+	24 ±11	33 ± 8	52 ± 8	-	-
II:5		16 ± 7	39 ± 2	48 ± 7	0.6-0.8	
III:1		19	40	62	-	-
III:2	A^+	30	52	91	-	-
III:4	O^+	26 ± 6	31 ± 4	48 ± 6	0.6-0.8	-
III:3	O^+	28 ± 21	63 ± 1	96 ± 27	1.4	
Family 4						
III:1	A^+	20 ± 7	26 ± 13	45 ± 19	0.6	0.6
II:1	O^+	28	35	39	1	1
II:2	A^+	64 ± 20	70 ± 22	131 ± 25	0.6	0.6
III:2	A^+	43 ± 11	58 ± 24	75 ± 22	0.6	0.8
Normal		50-150	45-145	60-150	0.8-1.2	≥ 1

vWF:Ag, antigenic von Willebrand factor. vWF:RCo, ristocetin cofactor. FVIII:C, factor VIII coagulant. RIPA, ristocetin-induced platelet agglutination (ristocetin concentration necessary to induce agglutination with an initial velocity of at least 20% agglutination); PRP, platelet-rich plasma of the patient; PPP, platelet-poor plasma and normal platelets. The mean and standard deviation is indicated when three or more determinations were carried out.[a] The multimeric assay revealed the lack of high and decrease of medium molecular weight multimers. This assay was normal in the other patients and their relatives.

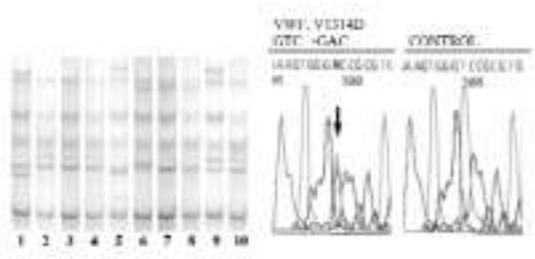

Figure 1. Detection of the 3941T→A (V1314D) mutation. The SSCP analysis of single chains of the 487 bp fragment digested with *DdeI* is shown on the left. Lanes 1-4: samples from family 1. Lanes 5-9: samples from patients with the V1316M, R1308C, R1306W, P1337P, and R1315R mutations. Lane 10: normal control.

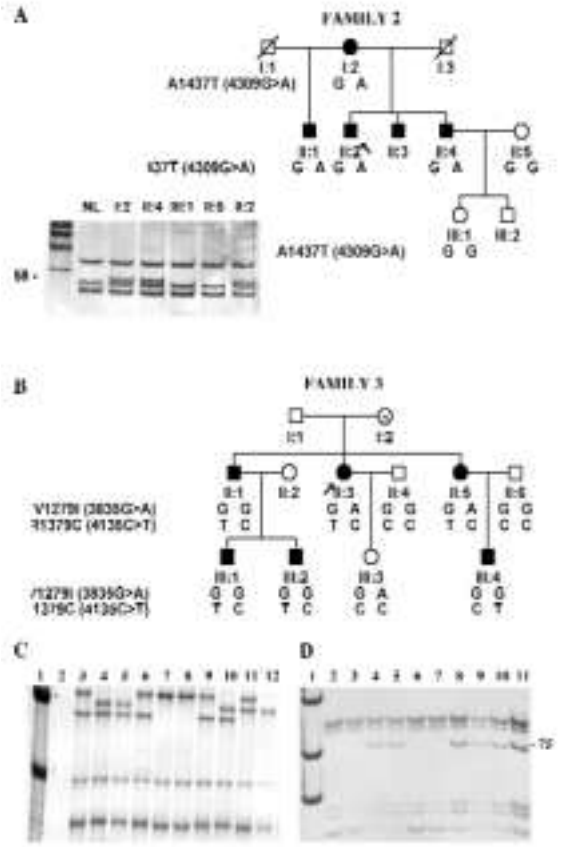

Figure 2. a) Family tree and detection of the 4309G→A (A1437T) mutation in family 2 with type 2M vWD. The filled symbols represent affected members. The digestion of a 294 bp fragment with *HaeIII* generates the next fragments: 4, 93, 52, 43(2), 15 and 43 bp in the normal alleles, and 4, 93, 52, 43(2) and 58 bp in the mutated alleles. The electrophoresis was carried out in polyacrylamide gel: T=20%, C=3.4%. b) Family tree (3). c) Electrophoresis in polyacrylamide gel (T=10%, C=3.4%) of the SSCP analysis of single chains of the 294 bp fragment. Lane 1: DNA marker 1-kb. Lane 2: non-denatured sample. Lanes 3-11: II:1, III:1, III:2, II:3, III:3, II:4, II:5, III:4, and II:6 samples from family 3. Lane 12: normal control for homozygous *HphI* marker (2). d) Detection of the 4135C→T (R1379) mutation by restriction analysis with *AciI*, and electrophoresis in polyacrylamide gel (T=15%, C=3.4%). The digestion of this 294 bp fragment generates the next fragments: 60, 15, 60 81 and 77 bp in normal alleles, and 60, 75, 81 and 77 bp in mutated alleles. Lane 1: DNA marker pBR322-*MspI* digest. Lane 2: normal control. Lanes 3-11: II:6, III:4, II:5, II:4, III:3, II:3, III:2, III:1 and II:1 samples. The extra 75 bp band generated in the mutated alleles can be seen in lanes 4-5 and 8-11.

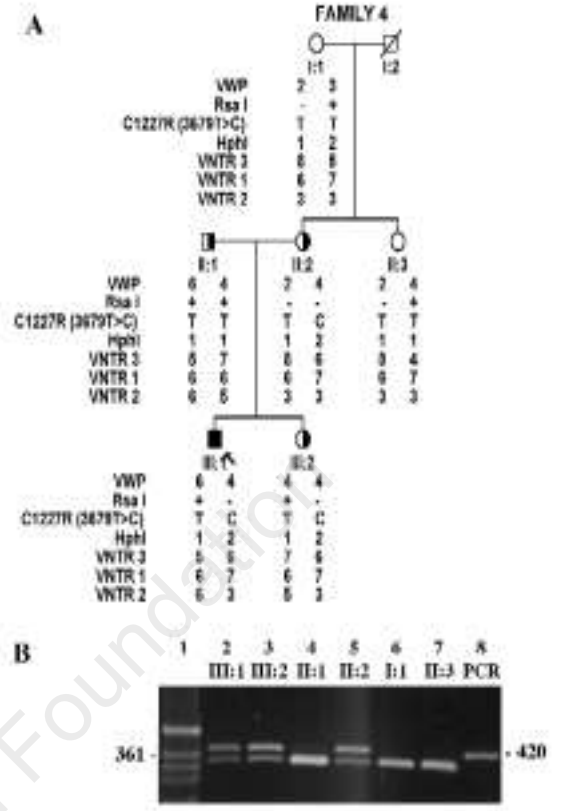

Figure 3. a) vWF gene polymorphism segregation analysis from family 4. The filled symbol represents the recessive type 1 vWD. Half symbols represent the heterozygous state. b) Detection of 3679T→C (C1227R) mutation by restriction analysis with AlwnI. The digestion of a 420 bp segment produces two of 361 and 59 bp. The small 59 bp band is not present in the 1.2% agarose gel.

using microfiltration (Centricon-100), and sequenced by the fluorescent dideoxy terminator method. The sequence analysis was carried out with the GCG program from Wisconsin Sequence Analysis Package.

The mutation nomenclature for vWF gene used in the present study is that recommended by the von Willebrand Factor Subcommittee of the Scientific and Standardization Committee of the ISTH.[6] For nucleotide changes, the numbering scheme is referred to the vWF cDNA sequence, from the A of the initiator ATG as site +1. For amino acid alterations, numbering starts from the initiator methionine as the +1 position.

Results

Single-stranded conformation polymorphism analysis from family 1 showed an anomalous pattern only in the patient, which indicated that a defect had arisen *de novo*. Automatic sequencing revealed the 3941T→A transversion in one allele, resulting in the amino acid change in which valine is substituted by aspartic at codon 1314 (V1314D) (Figure 1). This mutation was not detected in 110 normal alleles screened.

An abnormal electrophoretic migration of single chains was also observed in the SSCP analysis of the end fragment of the A1 domain from family 2, which segregates with the disease. The 4309G→A transition,

which predicts the change of alanine to threonine at codon 1437 (A1437T), was identified by sequencing in one patient. This mutation destroys an *HaeIII* restriction site, which enabled us to detect the mutation in the other patients (Figure 2a), and discard it in the 110 normal chromosomes studied.

A different anomalous pattern that segregated with the vWD phenotype was found in family 3 (Figure 2c). The mutation 4135C→T, which gives rise to a cysteine instead of the normal arginine at codon 1379 was identified (R1379C). This mutation, not previously described, destroys a restriction site for the *AciI* enzyme, which enabled us to detect it in the six patients (Figure 2d), and discard it in normal controls. Another change, (3835G→A), that predicts the substitution of valine by isoleucine at codon 1279 (V1279I), was detected in individuals II:3, II:5 and III:3 in the allele not associated with the disease (Figure 2b). The latter change was also present in the pseudogene sequence, and could be detected with *Sau3AI* because the enzyme recognises this sequence as a restriction site. A possible pseudogene contamination from genomic DNA could be excluded for two reasons. First, the polymerase chain reaction was gene-specific as checked by restriction analysis with *DdeI*, and second, the automatic sequencing only showed this change in heterozygous state, while no other pseudogene sequences were seen.

The family history, analytical data and polymorphism segregation analysis suggested that the propositus from family 4 (Table 1, Figure 3a) could be a compound heterozygote for defects in the vWF gene, and was classified as having type 1/2 vWD according to functional analysis. The nucleotide change 3679T→C, which predicts arginine in the protein instead of the normal cysteine, at the second codon of exon 28 (C1227R), was identified associated with the maternal allele, as checked by restriction analysis with *AlwnI* (Figure 3b). This substitution is close to the segment from C1237 to P1251, which is important for the interaction of vWF with platelet *GPIb*-IX[7]. The study of cDNA showed both alleles are expressed in the patient (data not shown).

Discussion

The V1314D mutation detected in the patient from family 1 has not been previously reported, although another change in the same codon (V1314L) was identified in one type 2B patient, who also had severe thrombocytopenia.[8] This mutation may be considered as a candidate for type 2B vWD for the following reasons. First, an uncharged amino acid (V) was substituted by another residue with a negative charge (D), which may affect the conformation of this region and increase the affinity of vWF for the platelet glycoprotein Ib. Second, the mutation is located in the region 1303-1341, where the majority of the defects causing this kind of functional variant are clustered. Third, the substitution has not been detected in normal controls. In the past, type 2B vWD was occasionally confused with ITP. Moreover, this variant has a similar phenotype to that of the pseudo-vWD. We therefore encourage physicians to check this possibility, at a genetic level if possible, to prevent the consequences that may arise from a wrong diagnosis.

Type 2M vWD refers to variants with decrease of platelet-dependent function in the presence of all vWF multimers. This type may include: i) some of the old type I forms, ii) variants with supra-normal multimers (vWD Vicenza), iii) types with few anomalies in their multimeric structure (type IC, ID), and iv) forms with the presence of the pro-vWF.[9-12] It is very easy to confuse type 2M vWD with type 1, because it can be difficult to detect discrepancies between antigenic and functional vWF, particularly when low protein levels are present in plasma, or the vWF:RCo is not evaluated correctly.[13] Type 2M can sometimes be confused with type 2A, because the main difference between them is the presence or absence of high molecular weight multimers.[4,14,15] The patients from family 2 were diagnosed a few years ago as having type I vWD. In our case, however, the mutation A1437T detected in these patients has been associated with the current variant 2M.

Patients II:3 and II:5 from family 3 were also originally diagnosed as having type 1 vWD. The analytical data from the patients from this family, who all had mild bleeding symptoms, normally showed few discrepancies between the functional and antigenic analyses. This could be because they have a functional variant, but the phenotype did not agree with any of the current subtypes and still remains unclassified, as do other mutations in this region.[16] This and the previously described case (family 2) are new examples of the difficulties clinicians can sometimes encounter when they attempt to diagnose vWD.[13,15] *In vitro* studies of plasma following infusion of the analogs of vassopressin (DDAVP) may help to clarify the question of whether the R1379C mutation gives a vWD phenotype with decrease of vWF levels and increase or decrease of platelet function. We have been unable to study the RIPA post-DDAVP because it was not possible for the patients to continue participating in the study. The V1279I mutation, which is also present in the pseudogene, has been associated with the allele that does not co-segregate with the phenotype in two of our patients. This change has also been reported in two patients with the P1266L mutation in the same allele. Both substitutions are present in the pseudogene sequence.[17,18] The authors propose a mechanism of inter-chromosomal conversion between the gene and the pseudogene, located respectively in chromosome 12 and 22, for this and other multiple adjacent nucleotide substitutions reflecting a small part of the vWF pseudogene sequence. The mutations resulting in a gene sequence that corresponds to the pseudogene have been registered in the database and are detailed as follows. The missense mutation G1609R causing type 2A,[19] and the nonsense mutation R1659X[20] have been recorded three and four times, respectively. Both occur in CG dinucleotides, which are considered hot spots for mutations, and have been detected in patients with type 2A and severe vWD

from different countries. Single mutations that do not take place at hot spots have also been identified, such as L1580P in type 2A,[21] the nonsense mutation Q1311X in type 3 VWD,[22] and F1369I associated with an unclassified variant.[23] Multiple substitutions have been detected in type 1 vWD patients: P1266 and V1279I,[18] and V1229G and N1231T,[24] both the result of a putative common gene conversion mechanism in this region. The V1279I change detected in three members from family 3 did not occur at a CG dinucleotide. There are two alternative mechanisms that could account for how this mutation may have arisen, namely a spontaneous mutational event or a gene conversion. In the latter case, a sequence from the pseudogene spanning no more than 135 bp would have been copied into the vWF gene. This length corresponds to the distance between the flanking pseudogene changes P1266L and Q1311X, which are not detected in the patient's vWF gene. It is not known how this substitution may influence the phenotype. In our study we observed this change in heterozygous state in the ten-year old girl (III:3) who had no history of bleeding and showed low ristocetin cofactor on some occasions (Table 1). There is a report of a patient who carried three pseudogene-like substitutions, including 3835G→A, in the heterozygous state and showed borderline vWF antigen and low ristocetin cofactor, but had no bleeding symptoms.[17] *In vitro* studies with the recombinant protein may help us to understand the effects that these candidate mutations produce. Other mutations in the vWF gene may modify the observed phenotypes, as a recent study points out,[25] but confirming this involves screening the rest of the gene, which is both costly and time-consuming. Nevertheless, this could be especially important for the patient from family 4 since it could be able to confirm whether the disease is caused by defects in the two vWF gene alleles or, by contrast, whether another locus is implicated in this phenotype.

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PC was responsible for designing the study, performing the genetic analyses, analyzing the data, and writing the manuscript. FM helped design the study and revise the manuscript. SH collected the clinical and analytical data from families 2-4, and AT the data from family 1. JAA supervised and revised the final version of the paper. All authors were actively involved in the discussion of the results. We wish to thank José Manuel Montoro for the multimeric structure and RIPA analyses, Carmen Espinós and Rafael Curats for their assistance in the indirect genetic analyses, and all the staff of Unidad de Coagulopatías Congénitas for their technical and clinical assistance, as well as Mr. Peter Blair for his linguistic advice about this paper.

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Potential implications for clinical practice

Detection of mutations associated with vWD phenotypes may allow more accurate genetic counselling.

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