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Evaluation of von Willebrand factor phenotypes and genotypes in Hemophilia A patients with and without identified F8 mutations

Brian Boylan¹, Anne S. Rice¹, Christine De Staercke¹, M. Elaine Eyster², Hassan M. Yaish³, Christine M. Knoll⁴, Christopher J. Bean¹, Connie H. Miller¹, and the Hemophilia Inhibitor Research Study Investigators*

¹Division of Blood Disorders, National Center on Birth Defects and Developmental Disabilities, Centers for Disease Control and Prevention, Atlanta, Georgia

²Department of Medicine, Division of Hematology and Oncology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania

³Department of Pediatrics, Division of Hematology/Oncology, University of Utah School of Medicine, Salt Lake City, Utah

⁴Center for Cancer and Blood Disorders, Phoenix Children's Hospital, Phoenix, Arizona

Summary

Background—Hemophilia A (HA) is an X-linked bleeding disorder caused by a deficiency in factor VIII (FVIII). von Willebrand disease (VWD) is characterized by a quantitative or qualitative defect in von Willebrand Factor (VWF). Patients with VWD with severely low VWF or VWD Type 2N (VWD2N), a VWD subtype distinguished by defective VWF binding to FVIII, may have reduced FVIII levels secondary to their VWD. These patients superficially resemble patients with HA, and pose a potential for misdiagnosis.

Objectives—Investigate the unexplained cause of bleeding in HA patients without known FVIII mutations by assessing plasma VWF antigen (VWF:Ag), FVIII binding capacities, and VWF genotypes.

Patients/Methods—Thirty-seven of 1027 patients with HA studied as part of the Hemophilia Inhibitor Research Study lacked identifiable *F8* mutations. These patients (cases) and 73 patients with identified *F8* mutations (controls) were evaluated for VWF:Ag, patient's VWF capacity to bind FVIII (VWF:FVIII_B), and VWF sequence.

Results—Four cases had VWF:Ag <3 IU/dL and VWF mutations consistent with Type3 VWD. Six cases and one control were heterozygous for mutations previously reported to cause Type1

Address correspondence to: Brian Boylan, Division of Blood Disorders, National Center on Birth Defects and Developmental Disabilities, 1600 Clifton Road, MS D-02, Atlanta, GA 30333, Phone: (404) 718-4031, Fax: (404) 639-1638, bboylan@cdc.gov.

*See Appendix for full list of Contributors.

Addendum: B.B. designed and performed the research, analyzed the results, and wrote the paper; A.S.R performed the research; C.D.S. performed the research and analyzed the results; M.E.E., H.M.Y., and C.M.K. provided patient samples and contributed to the manuscript; C.J.B designed the research, analyzed results, and contributed to the manuscript; C.H.M. designed the research, analyzed results, and wrote the paper.

VWD (VWD1) (n=5 cases and 1 control) or predicted to be deleterious by Polyphen2 and SIFT prediction tools (n=1 case). One control had VWF:Ag <30 IU/dl, and seven patients (4 cases and 3 controls), including two cases who were heterozygous for a known VWD2N mutation, had reduced VWF:FVIIIIB.

Conclusions—These data emphasize that some patients diagnosed with HA require VWF assessments in order to achieve a comprehensive diagnosis and an optimal treatment strategy.

Introduction

Although more than 2,500 *F8* mutations have been described(1), large Hemophilia A (HA) cohorts usually include a subset of patients for whom *F8* mutations are not identified(2;3). The differential diagnosis for HA includes von Willebrand disease (VWD), an autosomal bleeding disorder characterized by a deficiency in von Willebrand factor (VWF). VWF, a glycoprotein with a diverse set of hemostatic properties (reviewed in(4)), plays a pivotal role in recruiting platelets to sites of vascular injury and serves as a carrier protein for FVIII in plasma, acting both to stabilize bound FVIII and to sequester it from its procoagulant functions(5;6). Dissociation of FVIII from VWF is facilitated by cleavage of FVIII at three sites by thrombin, a process that also results in FVIII activation(7), and by the binding of VWF to exposed collagen at sites of vascular injury(8). Following its release from VWF, activated FVIII (FVIIIa) binds to anionic phospholipid surfaces where it forms a complex with activated Factor IX to promote Factor X activation(9) leading to downstream thrombin generation and clot formation. FVIIIa inactivation occurs by spontaneous dissociation of its A2 subunit(10) and via proteolysis by activated protein C(11), mechanisms that act together to guarantee FVIII's short plasma half-life when unbound to VWF.

Bleeding phenotypes in VWD are heterogeneous and most frequently include menorrhagia, bleeding from minor wounds, and (muco)cutaneous bleeding(12). Most forms of VWD can be distinguished from HA by measurement of VWF antigen (VWF:Ag) levels and ristocetin cofactor or other measures of VWF activity. VWD Types 1 and 3 (VWD1 and VWD3) manifest as partial and complete quantitative VWF deficiencies, respectively(4). Type 2 VWD (VWD2) is characterized by a qualitative defect that causes patients to express mildly reduced or normal levels of structurally abnormal VWF(4;13), including VWD Type 2N (VWD2N), which features a structural defect in VWF that impedes FVIII binding(14). In the absence of the stabilizing effects of VWF, patients with VWD3 and VWD2N homozygotes and compound heterozygotes have low plasma levels of FVIII(15), and, therefore present as clinically similar to patients with HA. The overlap of clinical features in these two patient groups can lead to persons with VWD being misdiagnosed as having HA(16;17) and potentially receiving suboptimal treatment.

In a large sample of patients with HA studied as part of the Hemophilia Inhibitor Research Study (HIRS) conducted by the Centers for Disease Control and Prevention (CDC), 37 (3.6%) of 1027 patients had no identified *F8* mutation. The current study examines plasma VWF antigen (VWF:Ag) and FVIII binding capacity (VWF:FVIIIIB) and analyzes *VWF* for coding mutations in these 37 cases in order to identify VWD patients misdiagnosed with HA.

Methods

Subjects—Patients with HA were enrolled in HIRS conducted at 17 U.S. hemophilia treatment centers between 2006 and 2013. The cohort in the current study (Table 1) includes and expands on an HA cohort previously described in a prospective surveillance study for inhibitors(18) and consists of 37 HA patients without identified *F8* exon or intron/exon boundary mutations (cases) and 65 consecutively enrolled, non-severe HA patients with known *F8* mutations (controls).

Quantitation of vWF binding capacity for FVIII—Blood was prepared and shipped to CDC as previously described (3). VWF:Ag was measured by latex immunoassay (Diagnostica Stago, Parsippany, NJ), and VWF:FVIIIIB was determined for plasma samples normalized for VWF:Ag using a commercially available enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Diagnostica Stago, Parsippany, NJ).

Molecular methods—Type O versus non-O blood groups were inferred by genotyping the frameshift-causing single base deletion in the *ABO* gene (rs8176719) using a TaqMan custom SNP assay as directed by the manufacturer (Applied Biosystems, Carlsbad, CA) and reported elsewhere(19). Each exon and +/- 20 base pairs of the adjacent intronic sequence of the *VWF* gene were targeted for next generation sequencing on an Illumina MiSeq system (Illumina, Inc., San Diego, CA). The presence of all pathological variants was verified by bi-directional Sanger re-sequencing using a 3730 DNA analyzer (Applied Biosystems, Carlsbad, CA).

Results and Discussion

Determination of HA patient VWF plasma levels

Plasma from 37 cases (HA patients with no known *F8* mutation) and 65 consecutively enrolled non-severe controls (HA patients with a known *F8* mutation) (Table 1) was tested for VWF plasma levels (VWF:Ag), and patients were classified according to National Heart Lung and Blood Institute (NHLBI) guidelines(20). As shown in Table 2, 4 cases (10.8%) had VWF:Ag below the 3 IU/dL threshold for a diagnosis of VWD3. One subject (control 1) had VWF:Ag in the range for presumptive VWD1 or VWD2 (3-30 IU/dL), and low VWF:Ag levels (30-50 IU/dL) were observed in 5 cases (13.5%) and 4 controls (5.5%).

Capacity of Patient VWF to bind FVIII

A commercially available ELISA was used to measure the capacity of each subject's plasma VWF to bind to recombinant FVIII. Four cases and 3 controls had VWF:FVIIIIB below 80% (Tables 2 and 4), which is the cutoff for healthy donors established by Veyradier et al.(21). Two of these four cases were heterozygous for p.R854Q (cases 10 and 13), a mutation reported to cause VWD2N (22). One case (case 9) was found to be heterozygous for a mutation identified in the 1000 Genomes Project(23) and predicted to be deleterious by Polyphen2(24) and SIFT(25) prediction tools (p.R2313C), while the other case did not have a detectable VWF mutation (case 15). The remaining 3 subjects with VWF:FVIIIIB <80% were controls, including one who was heterozygous for *VWF* p.Y1584C (control 1), a

mutation previously reported in patients with VWD1(22) and two others without a detectable *VWF* mutation (controls 11-12) (Tables 2 and 4).

Although both individuals with VWD2N mutations (cases 10 and 13) identified in the current study tested below the normal range for VWF:FVIIIIB, only one (case 13) had a result within the range established by Veyradier et al. for VWD2N heterozygotes (30-65%) (Table 2)(21). This discrepancy could be due to the fact that the VWD2N sample size used by Veyradier et al. to establish this range was relatively small (n=9) and may not accurately reflect the true range of VWF:FVIIIIB binding in VWD2N heterozygotes using this assay. An additional patient of interest, case 15, lacks an identified mutation in either *F8* or *VWF* and had VWF:FVIIIIB of 63%. The underlying reason for this patient's low VWF:FVIIIIB is unclear, but, in the Veyradier et al. study(21), a subset of patients with non-2N VWD or HA tested in this range. Future studies with a larger number of VWD2N heterozygotes are required to obtain a better estimate of the true VWF:FVIIIIB range. However, given the low number of patients available and because the presence of a single copy of a VWD2N mutation is unlikely to serve as the causative factor for a patient's bleeding diathesis, establishing, with confidence, a range for VWD2N heterozygotes may be of limited utility. It may be more practical to expand the range for possible VWD2N heterozygosity to include all samples that test below a normal reference group for VWF:FVIIIIB (<80%).

VWF genotypes of HA patients

Analysis of *VWF* gene sequence revealed that five patients had *VWF* sequence abnormalities that are known to cause a bleeding phenotype, including four cases who were homozygous or compound heterozygous for *VWF* nonsense or frameshift mutations. As shown in Table 3, case 3 has a homozygous frameshift mutation (p.P812Rfs*31) previously reported to cause VWD3(22), two patients (cases 1 and 2) are compound heterozygotes for previously reported *VWF* nonsense and frameshift mutations (p.R324X and p.P812Rfs*31), and one patient (case 4) is a compound heterozygote carrying p.W553Lfs*97(22) and p.A462Qfs*15 (not previously reported) *VWF* frameshift mutations. As expected, these four patients were the same four who had VWF:Ag levels <3 IU/dL (Table 2). One additional patient (Control 1), who has abnormally low VWF:Ag (28 IU/dL), is heterozygous for a *VWF* mutation previously reported to cause VWD1(22)(Tables 2 and 3).

Twenty-two additional patients who have less severe VWF abnormalities, which do not cause but may complicate the HA patient's bleeding phenotype, are shown in Table 4. Eight of these patients, including 5 cases (Cases 10-14) and three controls (Control 8-10), while having VWF:Ag 50 IU/dL, had mutations previously reported to contribute to some form of VWD(22), and three additional subjects (case 9 and controls 6-7) had *VWF* polymorphisms that are predicted to be deleterious by PolyPhen2(24) and/or SIFT(25) amino acid substitution prediction tools. Eight subjects without identified deleterious VWF polymorphisms (cases 5-8 and controls 2-5) had reduced VWF:Ag levels (30-50 IU/dL), (Table 4); however, it is notable that seven of these eight individuals are blood type O, which is known to correlate with decreased plasma levels of VWF(26).

Distinguishing VWD from HA has important implications for patient treatment strategies and provides patients with meaningful information regarding the genetics behind their

disorder. Our data reemphasize the conclusions previously published by Mazurier et al.(27) and Gupta et al.(28) by highlighting that VWF phenotypes are relevant in patients presenting with low FVIII. Overlooking this possibility may lead to suboptimal treatment of bleeding episodes in a subset of patients. The data presented herein demonstrate that there was a low frequency of patient referrals included in the HIRS study (four subjects; 0.4% of enrolled patients) who were misdiagnosed with moderate HA but actually had VWD3. Moreover, although one of these patients was receiving plasma derived FVIII, which contains residual VWF, the other three had a HA product treatment history consisting solely of recombinant FVIII at study entry. In the absence of supplemental VWF, the FVIII infusions received by these patients with VWD3 were unlikely to control all bleeding episodes adequately. An additional patient diagnosed with mild HA (control 1) with a known F8 mutation (p.2150C) was found to also carry a mutation previously reported to cause VWD1 (Y1584C)(22). This subject had VWF:Ag of 28 IU/dL and 13 U/dL of FVIII activity, and, despite his VWF deficiency, was on a HA treatment regimen consisting exclusively of recombinant FVIII. Overall, of the 14 patients identified to have reduced VWF, only 2 had a treatment history that included a product that would elevate plasma VWF. These data suggest that there is a subset of patients with HA with low VWF in whom post-infusion FVIII responses should be evaluated in order to determine if they may benefit from the use of products containing VWF.

The NHLBI guidelines suggest reserving the definite diagnosis of VWD for patients with VWF:Ag < 30 IU/dL and classifying individuals with levels of 30-50 IU/dL as “low VWF” (20). The current study identified nine case and control patients with mild to moderately reduced VWF:Ag (30-50 IU/dL). VWF levels are highly variable in healthy populations, and eight of the nine patients in our cohort who fall into the low VWF range have blood type O, which may account for the low expression levels. It is also possible that these patients have undetected *VWF* mutations or mutations in other gene(s) that affect VWF expression. The current study includes four patients (cases 5-8) in the low VWF range without an identified mutation in either *F8* or *VWF*. An off target mutation indirectly affecting VWF expression may contribute to the bleeding diathesis in these patients, particularly in subjects with considerable levels of residual FVIII activity (cases 5 and 7). In addition, as shown recently by Pezekshkpoor et al., low FVIII levels in HA patients without identified F8 mutations may be caused deep intronic mutations in *F8* that lead to alternative transcript splicing, which can cause frameshifts and low FVIII expression (29).

The current study identified five patients diagnosed with HA who have VWF mutations that play a causal role (Cases 1-4 and control 1) in their bleeding phenotype (Table 3). Twenty-two additional HA patients (11 Cases and 11 controls; Table 4) were found to have genetic, quantitative, or qualitative VWF abnormalities that, despite not serving as the primary cause for the patient's bleeding phenotype, highlight the importance of tailoring treatment strategies to individual patients. The inclusion of analyses of VWF phenotype and genotype to supplement standard *F8* analysis for patients diagnosed with HA will identify instances of misdiagnosis, but a proportion of patients may remain with no identified cause for their disease.

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Appendix

The Hemophilia Inhibitor Research Study Investigators

Thomas C. Abshire, Amy Dunn and Christine L. Kempton, Emory University, Atlanta, GA; Paula L. Bockenstedt, University of Michigan Hemophilia and Coagulation Disorders, Ann Arbor, MI; Doreen B. Brettler, New England Hemophilia Center, Worcester, MA; Jorge A. Di Paola, Mohamed Radhi and Steven R. Lentz, University of Iowa Carver College of Medicine, Iowa City, IA; Gita Massey and John C. Barrett, Virginia Commonwealth University, Richmond, VA; Anne T. Neff, Vanderbilt University Medical Center, Nashville, TN; Amy D. Shapiro, Indiana Hemophilia and Thrombosis Center, Indianapolis, IN; Michael Tarantino and Brian M. Wicklund, Kansas City Regional Hemophilia Center, Kansas City, MO; Marilyn J. Manco-Johnson, Mountain States Regional Hemophilia and Thrombosis Center, University of Colorado and The Children's Hospital, Aurora, CO; Miguel A. Escobar, Gulf States Hemophilia and Thrombophilia Center, Houston, TX; Joan C. Gill, Comprehensive Center for Bleeding Disorders, Milwaukee, WI; Cindy Leissingering, Louisiana Center for Bleeding and Clotting Disorders, New Orleans, LA.

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Table 1

Age and family history of bleeding data from Hemophilia A patients with or without known mutations.

Subjects	N	Mean Age at Study Entry (Median) [*]	Family History of Bleeding Disorder [†] %
Cases (Without <i>F8</i> Mutation)			
Severe HA	11	22.6 (15)	40
Non-Severe HA	26	22.8 (12) [*]	58.3
Controls (Without <i>F8</i> Mutation)			
Non-Severe HA	65	18.0 (11)	67.2

^{*} Age at study entry is unknown for 1 non-severe case

[†] Family history of bleeding is unknown for 4 controls, 1 severe case, and 2 non-severe cases.

von Willebrand factor measurements in study subjects. VWF:Ag= von Willebrand factor antigen. VWF:FVIII= factor VIII binding to von Willebrand factor VWD = von Willebrand disease.

Table 2

Subjects	n	VWF:Ag* (n with previously reported or predicted VWF mutation)		VWF:FVIII† (n with previously reported VWD2N mutation)		
		VWD Type 3 <3 IU/dL	VWD Type 1/2 3-30 IU/dL	Low VWF 31-50 IU/dL	VWD 2N homozygote or compound heterozygote <15%	VWD 2N heterozygote 30-65%
Cases (Without F8 Mutation)						
Severe HA	11	0	0	0	0	0
Non-Severe HA	26	4 (4)	0	5 (1)	0	2 (1)
Controls (With F8 Mutation)						
Non-Severe HA	65	0	1 (1)	4 (0)	0	3 (0)

* Ranges defined by National Heart, Lung, and Blood Institute (23)

† Ranges from Veyradier et al (24)

Table 3

Characterization of patients with von Willebrand factor mutations known to cause a bleeding phenotype. Low laboratory values are in bold.

Subject (Age in years at study entry)	VWF:Ag IU/dL	VWF:FVIIIB %	FVIII U/dL	F8 Mutation	VWF Mutation (allele number)	ABO Type	Treatment Product/exposure days
Case 1 (3)	1	NA	2	None	p.R324X [§] (1); p.P812Rfs*31 [§] (1)	Non-O	A/0-20
Case 2 (7)	1	NA	2	None	p.R324X [§] (1); p.P812Rfs*31 [§] (1)	Non-O	A/0-20
Case 3 (5)	2	NA	2	None	p.P812Rfs*31 [§] (2)	O	A/0-20
Case 4 (50)	2	NA	2	None	p.A462Qfs*15 [¶] (1); p.W553Lfs*97 [§] (1)	O	H/ND
Control 1 (2)	28	75	13	p.R2150C	p.Y1584C [§] (1)	O	A/0-20
Normal range	50-200	>80	60-160				

[§] Previously reported mutation known to cause some form of VWD.

[¶] A462Qfs*15 is a VWF frameshift mutation that has not previously been reported. A, Advate; H, Hemofil M; ND, no data

Table 4

Characteristics of patients with von Willebrand factor abnormalities that may contribute to bleeding phenotype in hemophilia A patients. Low laboratory values are in bold.

Subject (Age in years at study entry)	VWF:Ag IU/dL	VWF:FVIIIb %	FVIII U/dL	F8 Mutation	VWF Mutation (allele number)	ABO Type	Treatment Product/exposure days
Case 5 (2)	38	104	16	None	None	O	A/0-20
Case 6 (17)	42	108	6	None	None	O	A/>150
Case 7 (ND)	43	128	19	None	None	Non-O	R/ND
Case 8 (16)	50	128	4	None	None	O	X/>150
Case 9 (9)	50	77	4.5	None	p.R2313C* [†] (1)	O	R/0-20
Case 10 (6)	58	79	7	None	p.R854Q [§] (1)	Non-O	A/0-20
Case 11 (12)	59	100	3	None	p.P2063S [§] (1)	O	A/21-100
Case 12 (11)	68	108	5	None	p.M576I [§] (1)	Non-O	R/ND
Case 13 (10)	123	65	9	None	p.R854Q [§] (1)	O	A/ND
Case 14 (46)	183	118	< 1	None	p.P2063S [§] (1)	Non-O	AI/>150
Case 15 (47)	185	63	2	None	None	Non-O	R/0-20
Control 2 (3)	39	97	7	p.R1941Q	None	O	K/0-20
Control 3 (18)	43	96	14	p.E181G	None	O	N/0-20
Control 4 (2)	44	82	7	p.A593C	None	O	A/0-20
Control 5 (14)	50	109	1	p.R2163C	None	O	K/>150
Control 6 (36)	57	93	12	p.R1966Q	p.D1498N [‡] (1)	O	K/101-150
Control 7 (16)	75	117	5	p.R2307Q	p.R2575C [‡] (1)	Non-O	K/101-150
Control 8 (19)	89	130	3	p.R1781H	p.P2063S [§] (1)	Non-O	R/ND
Control 9 (3)	202	114	1	p.I1194F* ⁵	p.L129M [§] (1)	Non-O	A/>150
Control 10 (24)	228	105	36	p.S681P	p.P2063S [§] (1)	Non-O	R/0-20
Control 11 (22)	116	70	15	p.R593C	None	ND	ND
Control 12 (17)	292	72	23	p.R698W	None	ND	N
Normal Range	50-200	>80	60-160				

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* Identified in the 1000 genomes project (minor allele frequency 0.05%) (23).

[‡]VWF polymorphism predicted to be deleterious by Poly Phen 2 (24).

[‡]VWF polymorphism predicted to be deleterious by SIFT (25).

[§]Previously reported mutation known to cause some form of VWD.

A, Advate; AI, Alphanate; K, Kogenate; N, none; ND, no data; R, Recombinate; X, Xyntha