Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease

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To date, no dominant mutation has been identified in a significant proportion of patients with type 1 von Willebrand disease (VWD). In this study, we examined 70 families as part of the Canadian Type 1 VWD Study. The entire *VWF* gene was sequenced for 1 index case, revealing 2 sequence variations: intron 30 (5312−19A>C) and exon 28 at Tyr1584Cys (4751A>G). The Tyr1584Cys variation was identified in 14.3% (10 of 70) of the families and was in phase with the 5312−19A>C variation in 7 (10.0%) families. Both variants were observed in 2 of 10 UK families with type 1 VWD, but neither variant was found in 200 and 100 healthy, unrelated persons, respectively. Mean von Willebrand factor antigen (VWF:Ag), VWF ristocetin cofactor (VWF:RCo), and factor VIII coagulant activity (FVIII:C) for the index cases in these families are 0.4 U/mL, 0.36 U/mL, and 0.54 U/mL, respectively, and VWF multimer patterns show no qualitative abnormalities. Aberrant VWF splicing was not observed in these patients, and both alleles of the *VWF* gene are expressed as RNA. Molecular dynamic simulation was performed on a homology model of the VWF-A2 domain containing the Tyr1584Cys mutation. This showed that no significant structural changes occur as a result of the substitution but that a new solvent-exposed reactive thiol group is apparent. Expression studies revealed that the Tyr1584Cys mutation results in increased intracellular retention of the VWF protein. We demonstrate that all the families with the Tyr1584Cys mutation share a common, evolved VWF haplotype, suggesting that this mutation is ancient. This is the first report of a mutation that segregates in a significant proportion of patients with type 1 VWD. (Blood. 2003;102:549-557)

Introduction

Studies in the past 2 decades have confirmed that the most common inherited bleeding disorder in humans is von Willebrand disease (VWD). Prevalence rate estimates between 0.01% and 1% in the population have been proposed based on varying diagnostic definitions.[1-5] The 3 criteria required for a diagnosis of type 1 VWD are history of excessive mucocutaneous bleeding, quantitative or qualitative abnormalities of von Willebrand factor (VWF), and family history of the disorder. In general, type 2 VWD, in which qualitative mutant forms of VWF are present, and type 3 VWD, which includes a complete absence of VWF from the plasma, present few, if any, problems in diagnosis. In contrast, type 1 VWD, which is the most frequent form of the disease and occurs in approximately 80% of all VWD patients, is characterized by mild to moderate reductions in normal plasma VWF levels. Type 1 VWD presents a frequent diagnostic dilemma and often requires repeated laboratory testing before a definitive conclusion can be reached.

The inheritance pattern of types 1 and 2 VWD is autosomal dominant, whereas the inheritance pattern of the rare, severe type 3 form of the disease is recessive. Since the cloning of the *VWF* gene in 1985,[6-9] significant progress has been made in characterizing the molecular genetic pathology associated with several of the type 2 mutants, and different VWF null alleles have been documented in type 3 VWD.[10-14] In some cases, such as those associated with large *VWF* gene deletions, treatment with VWF-containing plasma concentrates has been complicated by the development of an anti-VWF antibody response.[15,16]

In contrast, progress in defining the molecular genetic basis of type 1 VWD has been slow and frustrating. Indeed, studies in a mouse model of type 1 VWD have indicated that locus heterogeneity may be associated with this condition and that defects in genes involved in various aspects of the VWF biosynthetic pathway may play a role in at least some of the cases of this purely quantitative trait.[17,18] Nevertheless, the *VWF* gene itself has remained the primary candidate location for the sequence variation responsible for the reduced amount of VWF seen in type 1 VWD. There have been a few sporadic reports of "candidate" type 1 mutations, some of which represent the heterozygous inheritance of VWF null

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A complete list of the members of the Association of Hemophilia Clinic Directors of Canada appears in the "Appendix."

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alleles.[19] To address the molecular basis of type 1 VWD in a more comprehensive manner, a Canadian, population-based study was recently initiated. In this article, we report, for the first time, a recurring VWD mutation, Tyr1584Cys, that associates with a common haplotype in a significant proportion of patients with type 1 VWD.

Patients, materials, and methods

Patients

The study population comprised 70 families from the Canadian Type 1 VWD Study (samples collected in collaboration with the Association of Hemophilia Clinic Directors of Canada [AHCDC]). Sixty-four percent of families are of mixed ethnicity from the province of Quebec, and the remaining families are from other provinces in Canada. Ten additional families were found through the Haemophilia Centre at the University of Southampton in the United Kingdom. All participants in this study were informed about the experimental nature of the study and gave their consent to take part. This study was approved by the Institutional Review Board at Queen's University and at each of the source institutions. In the 12 families included in this study, at least one member received a diagnosis of type 1 VWD (index case) because of a personal history of excessive mucocutaneous bleeding, von Willebrand factor antigen (VWF:Ag), and ristocetin cofactor activity (VWF:RCo) between 0.05 and 0.50 U/mL measured on at least 2 different blood samples. Whole blood samples for DNA extraction were collected by phlebotomy in 3.2% sodium citrate (at a ratio of 9:1 vol/vol) from the index case and from available immediate family members, and a plasma sample was collected from the index case for repeat VWD phenotypic studies to confirm the diagnosis of type 1 VWD.

Coagulation studies

Laboratory tests for VWF:Ag, VWF:RCo, and factor VIII coagulant activity (FVIII:C) were performed at the source clinic attended by the patient. These tests were repeated on different plasma samples at the Clinical Hemostasis Laboratory at Kingston General Hospital (Canada), and the 2 sets of test results were averaged. VWF:Ag was measured by the IMUBIND VWF enzyme-linked immunosorbent assay (ELISA) kit according to the procedure supplied by the manufacturer (American Diagnostica, Greenwich, CT). The VWF:RCo was measured by platelet aggregometry using freshly prepared, washed normal platelets,[20] and FVIII:C was measured using a 1-stage assay.[21] All measurements of VWF:Ag, VWF:RCo, and FVIII:C were made against a non–ABO-matched commercial reference plasma that had, in turn, been calibrated against the 91-666 or 97-586 World Health Organization (WHO) Plasma Standard. VWF multimers were analyzed by electrophoresis using a 1.6% sodium dodecyl sulfate (SDS) agarose gel followed by electrotransfer to a nylon membrane, and the multimers were visualized using the chemiluminescent visualization kit from Amersham Pharmacia Biotech (Baie D'Urfe, PQ, Canada).[22]

PCR amplification of genomic DNA and mutation identification

A blood sample was collected from the patient, and genomic DNA was isolated from leukocytes using a salt extraction method.[23] DNA corresponding to the coding sequence of exons 2-52 of VWF and the adjacent exon/intron boundaries and branch sites was amplified by polymerase chain reaction (PCR). Some primer sequences of interest are found in Table 1; other primer sequences are available on request from the authors. Using a DNA thermal cycler (Perkin Elmer Life Sciences, Shelton, CT), DNA was amplified for 35 cycles of 45 to 60 seconds at 94°C, 45 to 60 seconds at 53°C to 60°C, and 45 to 60 seconds at 72°C, and the amplified products were sequenced directly on an ABI model 373 automated sequencer (Cortec DNA Service Laboratories, Kingston, ON, Canada). All DNA sequences were compared with normal human *VWF* DNA sequences with the assistance of Vector NTI Suite software (InforMax, Bethesda, MD). Canadian blood group O donors were used as a source of normal DNA.

RNA isolation and amplification

Cytoplasmic RNA was isolated from the patient's platelets using TRIzol reagent (Invitrogen, Burlington, ON, Canada) and was reverse transcribed

Table 1. Primers for *VWF* gene amplification, site-directed mutagenesis, and construction of pBKVWF29-31 (C and A)

Primer, F/R	Purpose	Sequence, 5′ > 3′	Location
VWF-HS-nuc802-F (promoter)	SNP analysis	ATAAGAGCTGGAAGTGGAAA	1-802
VWF-HS-prom905-R (promoter)	SNP analysis	AACCTCCTCCCTTCCCACATA	1-1276
8F	SNP analysis	GGTAAGGGCCTCACAAGAT	5-39
8R	SNP analysis	GTGCTGGCAAGGTCTCTGA	5-420
12F	SNP analysis	TTGAGGCCTTTCTCTGATTAA	9-4
12R	SNP analysis	TGCTAAGGGATGGGCTGTG	9-288
13F	SNP analysis	GCTACCATCCTTTTGAGACAC	10-20
13R	SNP analysis	CACCACACAAAGCCATTCTAC	10-312
18F	SNP analysis	GGTCCATTATCTCCTTCACT	15-159
18R	SNP analysis	CCTGCCTACAAGAAAACT	15-423
28AF	SNP analysis	CAGAAGTGTCCA**C**AGGTTCTT**C**	24-149
28BR	SNP analysis	GCAGGATTTC**C**GGTGAC	24-1366
28CF	SNP analysis	CCTGA**A**GCCCCTCCTCCTACT	24-915
28DR	SNP analysis	AGGATTAGAACCC**G**AGTC**G**	24-1684
29F	SNP analysis	**A**TTGCCCTTG**T**ACTCAC**G**	10335*
31R	SNP analysis	ATCCAAAAGTAACCCCAGG**C**	11340*
42F	SNP analysis	GCACCCTATAGCATAGCTGA	31-3610
42R	SNP analysis	ATAGTTAATAGCCAAGCAGT	31-4227
Sac I exon 29	Plasmid construction	GAGCTCACTGCAGCCAGCCCCTGGAC	25-44
Spe I exon 32	Plasmid construction	ACTAGTCAGAGCACAGTTTGTGGAGGA	26-435
7912F	cDNA amplification	GCTCCCACGCCTACATC	24-502
99R	cDNA amplification	TGAAAGCCTT**G**GC**G**AAACTCT	25-42
10767F	cDNA amplification	AACGTGG**T**CCCGGAGAAA	10767*
VWFex32nt91-R	cDNA amplification	TTCGATT**C**GCTGGAGCTTCA	26-380
HS-VWF-nt4740-mutagenic G	SDM	CTGGCCCTGCGGT(G)CCTCTCTGACCAC	24-1264

Sequence mismatches with the VWF pseudogene are indicated in bold and polymorphisms found within the VWF gene are indicated in italic; underlined sequences are engineered restriction enzyme sites. Nucleotide changed in site directed mutagenesis primer is in brackets. Locations of primer are according to Mancuso,[24] unless indicated by* (numbering from[25]). F indicates forward primers; R, reverse primers; and SDM, site directed mutagenesis.

according to the procedure supplied by the manufacturer (Expand Reverse Transcriptase; Roche, Laval, PQ, Canada). The cDNA was PCR amplified from exons 28-29 (primers 7912F and 99R) and exons 30-32 (primers 10767F and 32nt91R), and the resultant PCR product was sequenced.

Genotype analysis

A VWF haplotype was derived from restriction enzyme digests of PCR-amplified fragments of the *VWF* gene and from the number of microsatellite repeats found at STR 2 within intron 40. SNPs that were included in the haplotype included 2 promoter polymorphisms at nucleotides −1185 (G+/A−, *Bst*UI) and −1051 (A+/G−, *Nla*III), exon 8, aa 318 (T+/A−, *Msl*I), exon 12, aa 471 (G+/A−, *Aat*II), exon 13, aa 484 (G+/A−, *Rsa*I), exon 18, aa 789 (A+/G−, *Rsa*I), exon 28, aa 1547 (C+/T−, *Bst*EII), exon 28, aa 1584 (A+/G−, *Kpn*I), intron 30, nt −18 (A−/C+, *Bsp*1286I), exon 42, aa 2413 (C+/T−, *Aci*I), and STR 2 in intron 40.[26]

Site-directed mutagenesis, plasmid construction, expression, and characterization

To assess whether the 5312−19A>C sequence variation induces abnormal splicing, genomic DNA was PCR amplified (Expand Long Template PCR System; Roche) from the first nucleotide of exon 29 (nt 5054, in cDNA sequence) to the last nucleotide of exon 32 (nt 5620, in cDNA sequence) using the primers *Sac*I exon 29 and *Spe*I exon 32, which have *Sac*I and *Spe*I restriction sites engineered at the 5′ end of the primers, respectively (Table 1). The 3390-bp PCR product was subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and the sequence between intron 30 and exon 31 was sequenced to confirm the integrity of the amplified product. pCR2.1 was digested with *Bsp*1286I to identify plasmids that contained the A (pCR2.1A) and C (pCR2.1C) nucleotides at intron 30 (5312−19A>C). Constructs pCR2.1A and pCR2.1C and the expression vector pBK-CMV (Stratagene, La Jolla, CA) were digested with *Sac*I and *Spe*I, and the excised sequence from exons 29-32 was cloned into pBK-CMV to generate pBKVWF29-32A and pBKVWF29-32C.

To assess the effect of the Tyr1584Cys sequence variation, site-directed mutagenesis was performed on the pCIneoVWFES expression vector (kindly provided by Dr P. Kroner, Medical College of Wisconsin, Milwaukee). A unique *Eco*RI restriction site was created by Dr Kroner in exon 28 at codon 4476 of the *VWF* cDNA. This *Eco*RI site, in conjunction with the unique *Not*I site present in the multicloning region of pCIneoVWFES, was used to excise the 3′ end of the *VWF* gene that was subsequently subcloned into the multicloning site of Bluescript to produce the vector Bluescript VWF *Eco*RI-*Not*I. To introduce the A>G transition at nucleotide 4751, site-directed mutagenesis was performed on Bluescript VWF *Eco*RI-*Not*I using the QuikChange MultiSite-Directed Mutagenesis kit (Stratagene) according to the manufacturer's procedures (primer listed in Table 1). The mutant product was sequenced to verify the presence of the Tyr1584Cys substitution and to confirm the integrity of the amplified *VWF* gene. The *Eco*RI-*Not*I mutant fragment was excised from Bluescript and ligated back into pCIneoVWFES to produce the vector pCIneoC1584, and plasmid DNA was purified for transfection.

COS-7 cells (catalog no. CRL-1651; American Type Culture Collection, Rockville, MD) were cultured in Dulbecco modified Eagle medium (DMEM) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) at 37°C in 5% CO_2. Cells in the log phase of growth were seeded at a density such that cells were approximately 50% confluent the following day. To assess the intron 30 (5312−19A>C) variant, these cells were transfected with 20 μg pBKVWF29-32A, 20 μg pBKVWF29-32C, or 10 μg each of pBKVWF29-32A and pBKVWF29-32C using calcium phosphate.[27] Forty-eight hours after transfection, RNA was extracted with TRIzol reagent and was reverse transcribed using an oligo-dT primer. cDNA was PCR amplified with primers *Sac*I exon 29 and *Spe*I exon 32 to generate a 567-bp fragment that was subsequently sequenced.

To assess the expression of the Tyr1584Cys variant, COS-7 cells were transfected with 20 μg DNA (10 μg pCIneoVWFC1584 plasmid, 10 μg pCIneoVWFY1584 plasmid, or 5 μg each of these plasmids; 3.2 μg βgal reporter construct, and 6.8 μg calf thymus DNA) using the method outlined above. Forty-eight hours after transfection, media were collected and cells were lysed. Transfection efficiency was determined for each experiment by measuring βgal activity from the βgal reporter transcript using a Berthold Lumat LB 9501 luminometer (Fisher Scientific, Springfield, NJ) and the Galacto Light Plus reporter gene assay (Tropix, Bedford, MA). The quantity of recombinant VWF:Ag present in the media and cell lysates was determined by ELISA using a polyclonal goat antihuman VWF antibody (Affinity Biologicals, Hamilton, ON, Canada). The standard curve for the ELISA was generated using CryoCheck normal human reference plasma (lot no. 7070; Precision Biologicals, Dartmouth, NS, Canada).

Molecular modeling of the Tyr1584Cys variant

A 3-dimensional homology model of the VWF A2 domain has been developed in our laboratory, based on the crystallographic structures of homologous domains from the integrins α1-β1, α2-β1, αL, and αM and from the VWF A1 and A3 domains (J.J.S., manuscript in preparation). After the replacement of tyrosine with cysteine, a molecular dynamic simulation was performed to assess the structural change caused by the mutation. A 10-nanosecond trajectory of the wild-type and mutant proteins at physiological conditions was obtained with the program NAMD.[28]

Results

Phenotype

Seventy families with at least one member who received a diagnosis of type 1 VWD were examined for the Tyr1584Cys mutation. As we have suggested, there is no consensus regarding a diagnostic definition for type 1 VWD. In this study, we used diagnostic criteria in which each of the 12 index cases with the Tyr1584Cys mutation has a personal history of excessive mucocutaneous bleeding and for whom VWF:Ag and VWF:RCo levels were documented on at least 2 occasions to be higher than 0.05 U/mL and lower than 0.5 U/mL. Although our study definition of type 1 VWD did not require a documented family history of the disorder, many families in the study have several members with diagnoses of type 1 VWD, which is consistent with a dominant mode of inheritance. Hemostatic test results from index cases were calculated from an average of at least 2 sets of studies; separate samples were analyzed at the source laboratory at which the sample was drawn and at the Clinical Hemostasis Laboratory at Kingston General Hospital. Clinical symptoms and plasma levels of VWF:Ag, VWF:RCo, and FVIII:C for the index cases that have the Tyr1584Cys mutation are summarized in Table 2, and the mean levels for the index cases are 0.40 U/mL, 0.36 U/mL, and 0.54 U/mL, respectively. It is of note that the ABO blood group in all but one of these index cases is type O. The association of lower VWF levels with type O blood group has been well established, and there is evidence that approximately 60% of the heritability of plasma VWF levels is linked to this genetic locus.[29] The FVIII:C level of the index case from family 17 was significantly lower than the FVIII levels in our other index cases, but DNA sequencing of exons encoding the VWF FVIII binding domain revealed no sequence variations previously associated with type 2N VWD. Whether this patient has coinherited a mild hemophilia A allele or has a novel FVIII binding mutation at another location in the *VWF* gene remains to be resolved. Plasma VWF multimers on index cases

Table 2. Laboratory and clinical phenotypic data for propositi in type 1 families with the Tyr1584Cys mutation

Family no.	VWF:Ag, U/mL	VWF:RCo, U/mL	FVIII:C, U/mL	Blood group	Sex	Symptoms
2	0.33	0.36	0.62	A	F	a, d
11	0.47	0.38	0.51	O	F	a, b, d
17	0.36	0.32	0.11	O	M	a, b, c, e, f
31	0.33	0.43	0.37	O	F	d, e, f
33	0.47	0.29	0.44	O	M	b, c, e, f
34	0.50	0.31	0.67	O	F	b
49	0.40	0.32	0.71	O	M	b, c
59	0.47	0.44	0.40	O	F	b
66	0.37	0.37	0.56	O	M	c
70	0.42	0.46	1.01	O	F	a, c, d
A*	0.44	0.32	0.74	O	F	b, d, e
I*	0.24	0.36	0.38	O	M	a, b, f
Mean	0.40	0.36	0.54	—	—	—

a indicates easy bruising; b, epistaxis; c, prolonged bleeding from wounds; d, menorrhagia; e, postoperative bleeding; f, bleeding after dental procedure; and —, not applicable.

*Families from the United Kingdom.

yielded all sizes of multimers at reduced intensity, which is consistent with the decreased plasma levels of VWF observed in patients with type 1 VWD. All the index cases exhibited a bleeding history with one or more of the following symptoms: epistaxis, menorrhagia, easy bruising, and excessive bleeding from wounds, especially after surgery and dental procedures (Table 2).

Sequence variations

DNA corresponding to the coding sequence of the *VWF* gene from exons 2-52 or 18-52, including exon/intron boundaries and the intron branch sites, was PCR amplified and sequenced for the index cases from families 2 and 34, respectively. Families 2 and 34 were selected for sequence analysis because the index cases in these families had a clear type 1 phenotype, and the samples from these patients were among the first we received that had the Tyr1584Cys mutation. Sequence analysis revealed an additional A>C transversion in intron 30 (5312−19A>C); aside from common polymorphisms, no other sequence variations were observed in either patient.

The A>C transversion in intron 30 is located 19 nucleotides upstream of exon 31. This sequence variation has previously been recorded as a rare polymorphism in the VWF database (http://www.shef.ac.uk/VWF/) with a frequency of 1%, and it creates a restriction enzyme site for *Bsp*1286I. Restriction enzyme digest analysis was used to confirm that both patients were heterozygous for the nucleotide change (Figure 1A). This sequence variation was not detected in 200 healthy persons (400 *VWF* alleles); however, it was identified in both affected sons from family 2 and the mother from family 34. This sequence variation was also found in the heterozygous state in affected or undiagnosed members of 5 additional families from the Canadian Type 1 VWD Study. The frequency of this variation is 0% in the healthy population and 10% (7 of 70) in the Canadian type 1 VWD families (16 of 150 [10.7%] affected persons).

The A>G transition in exon 28 at nucleotide 4751 (4751A>G), which results in the nonconservative amino acid substitution of Tyr1584Cys, was also found. This sequence variation has been recorded as a polymorphism in the VWF database, with a frequency of 2%, and it creates a restriction enzyme site for *Kpn*I. Restriction enzyme digest analysis was used to confirm that both patients were heterozygous for the nucleotide change (Figure 1B). This sequence variation was detected in every participant with the intron 30 sequence variation (5312−19A>C) and in affected family members from 3 additional families. To assess the general population frequency of this change, DNA from 100 healthy persons was examined. The sequence variation was found in one person; however, this person may have undiagnosed type 1 VWD because the plasma VWF:Ag level was found to be 0.5 U/mL on the single sample available. If this person does have VWD—and as an encrypted sample from a blood donor population, it is impossible to confirm or refute this possibility—the frequency of this variation is 0% in the healthy population tested and 14.3% (10 of 70) in the Canadian Type 1 VWD Study families (21 of 150 [14.0%] affected participants). Several of the family members who have the Tyr1584Cys mutation have not had diagnoses of type 1 VWD. They do, however, have prolonged in vitro bleeding times or are unavailable or uninterested in further diagnostic testing. One participant in this study with the Tyr1584Cys mutation has a normal VWF:Ag level and no bleeding history. This demonstrates that the Tyr1584Cys mutation does not have complete penetrance. Analysis of 10 families with type 1 VWD, ascertained at the Haemophilia Centre in Southampton, United Kingdom, revealed the coexistence of the 5312−19A>C and 4751A>G sequence variations in 2 families. One member from 1 of these families, with a significantly lower VWF:Ag level of 0.24 U/mL, was homozygous for the 4751A>G sequence variation.

RNA analysis for the 5312−19A>C sequence variation

The 5312−19A>C sequence variation is located at a putative consensus branch site sequence 19 nucleotides upstream of exon 31 (Figure 2A). The consensus sequence for a splicing branch site is Py_{80} N Py_{80} Py_{87} Pu_{75} A_{100} Py_{95}, where Py is pyrimidine and Pu is purine; the numbers indicate the percentage of time that particular base occurs in that position in the consensus sequence.[30] The second-to-last nucleotide in this consensus sequence is an invariant A and is essential for correct splicing. The 5312−19A>C sequence variation is located at this invariant A in a putative branch site consensus sequence (GCGTGA/CT); therefore, it was postulated that the 5312−19A>C transversion might result in abnormal splicing. To examine the possibility of abnormal splicing, RNA isolated from the platelets of the index case of family 2 and of a

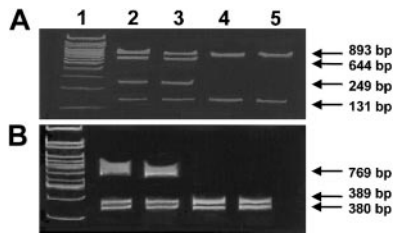

Figure 1. Restriction enzyme digest analysis for the 5312−19A>C and 4751A>G sequence variations from family 17. (A) The VWF DNA from exons 29-31 was PCR amplified with primers 29F and 31R. *Bsp*1286I cuts the wild-type PCR product once, yielding bands of 893 bp and 131 bp, as is observed in the unaffected members in lanes 4 and 5. The 5312A>C sequence variation creates an additional *Bsp*1286I restriction enzyme site cutting the 893-bp band once, yielding 2 fragments of 644 bp and 249 bp. Four bands of 893 bp, 644 bp, 249 bp, and 131 bp are observed in the members heterozygous for this change (lanes 2, 3). (B) The 4751A>C mutation in exon 28 (amplified with primers 28CF and 28DR) destroys a restriction enzyme site for *Kpn*I. Two bands of 389 bp and 380 bp were observed in the unaffected members (lanes 4, 5), and 3 bands of 389 bp, 380 bp (cut), and 769 bp (uncut) were observed in the affected members who are heterozygous for this change (lanes 2, 3). Lane 1 is a 100-bp ladder.

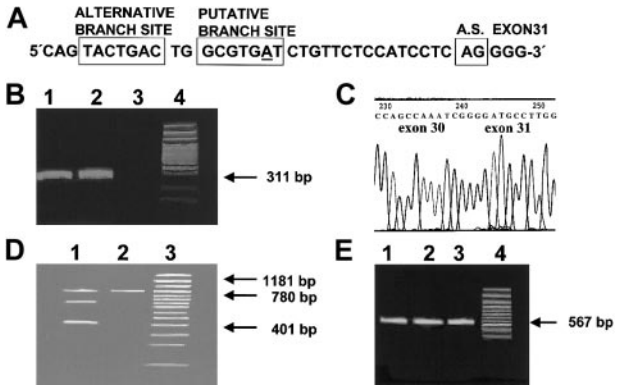

Figure 2. RNA analysis for 5312−19A>C sequence variation. (A) DNA sequence from intron 30 and exon 31 showing the putative branch site, an alternative branch site, and the acceptor splice (AS) site. The 5312−19A>C sequence variation is underlined in the putative branch site. (B) RNA isolated from the platelets of the index case of family 2 and from a healthy person was reverse transcribed and PCR amplified from exons 30-32. Only one *VWF* cDNA product of 311 bp was observed in both persons, indicating correct splicing (lane 1, patient; lane 2, healthy person; lane 3, PCR blank; lane 4, 100-bp ladder). (C) Sequence analysis revealed a correctly spliced product, with exon 30 spliced directly to exon 31. (D) RNA from the index case of family 2 and a healthy person was reverse transcribed and PCR amplified from exons 28-29 (amplified product contains aa 1584). The PCR product was subsequently digested with *Kpn*I, and 3 bands of 1181 bp (uncut), 780 bp, and 401 bp were observed for the patient, demonstrating that both alleles are stably expressed as RNA (lane 1). Only one band is observed in the healthy person because he does not have the mutation (lane 2). Lane 3, 100-bp ladder. (E) RNA isolated from COS-7 cells transfected with pBKVWF29-32A, pBKVWF29-32C, or both (which contain either the A or C at 5312) was reverse transcribed. Only one VWF product of 567 bp was amplified from the wild-type (lane 1), cotransfected (lane 2), or homozygous sequence variant (lane 3) cDNAs. Lane 4 is a 100-bp ladder.

healthy person was reverse transcribed and PCR amplified. Only one VWF cDNA product of 311 bp was observed in each, suggesting that abnormal splicing did not occur in the index case (Figure 2B). Furthermore, sequence analysis revealed a correctly spliced product, with exon 30 spliced directly to exon 31 (Figure 2C). A potential explanation for these findings is that an aberrantly spliced product is produced by this variant allele but is unstable and rapidly degraded and is, therefore, not observed in RNA prepared from platelets. However, amplified cDNA from exon 28 revealed that both alleles are expressed as RNA in the patient's platelets (Figure 2D).

To further assess the potential pathogenic role of the 5312−19A>C variant, COS-7 cells were transiently transfected with the pBKVWF29-32A and pBKVWF29-32C constructs, which contain either A or C at nt 5312. Forty-eight hours after transfection, RNA was extracted from the cells, reverse transcribed, PCR amplified, and sequenced. Only one VWF PCR product of 567 bp was amplified from the cDNA of COS-7 cells transfected with the wild-type (A), sequence variant (C), and cotransfected (A and C) constructs (Figure 2E).

Expression and characterization of recombinant Tyr1584Cys VWF

To determine the effect of the Tyr1584Cys substitution on VWF biosynthesis, site-directed mutagenesis was used to introduce the substitution to an expression vector containing the full-length *VWF* cDNA. The expression vectors pCIneoY1584 (wild-type) and pCIneoC1584 (mutant) were transiently transfected into COS-7 cells alone or together to generate wild-type, mutant, and heterozygous genotypes. Transfection efficiencies for the mutant and heterozygous transfections (as determined by βgal activity) were normalized to the wild-type transfection. To determine whether the mutant recombinant protein was retained within the cell or was efficiently secreted, VWF:Ag levels were assayed in cell lysates and in the conditioned media using ELISA; the data are presented in Figure 3. Secretion of rVWF protein from COS-7 cells transfected with the pCIneoC1584 construct or cotransfected with both constructs (heterozygous state) was decreased by 38% ($P = .005$) and 16% ($P = .07$), respectively, relative to the secretion of wild-type rVWF protein (VWF:Ag levels for wild-type, heterozygous, and mutant are 2.681 ± 0.122 U/mL, 2.244 ± 0.168 U/mL, and 1.649 ± 0.250 U/mL, respectively; n = 4-6). Intracellular levels of rVWF were increased by 47% ($P = .04$) and 8% ($P = .6$), respectively (VWF:Ag levels for wild-type, heterozygous, and mutant are 1.735 ± 0.167 U/mL, 1.867 ± 0.141 U/mL, and 2.592 ± 0.320 U/mL, respectively). These results demonstrate that the Tyr1584Cys mutation results in increased intracellular retention of the protein.

Molecular modeling of the Tyr1584Cys variant

Molecular modeling was used to assess the effect of the Tyr1584Cys mutation on protein structure. A homology model of the VWF A2 domain indicated that the cysteine residue is located on alpha helix 4, at the surface of the protein, and the reactive thiol (SH) side chain on the substituted cysteine is 100% exposed to solvent. A 10-nanosecond molecular dynamics simulation of the mutant protein indicated that the replacement of tyrosine by cysteine causes no significant structural changes (at the end of the simulation, Tyr1584Cys has a root-mean-square deviation for α-carbons of 2.0 Å from the starting structure, compared with 2.3 Å for the wild-type model [J. J. S., manuscript in preparation]), and the thiol group remains fully exposed to solvent (Figure 4).

VWF haplotype generation

A polymorphic haplotype was generated to determine whether the 2 sequence variations (5312−19A>C, 4751A>G) are in phase and whether all affected family members with these sequence variations have a common founder haplotype. Nine SNPs located throughout the *VWF* gene and 1 microsatellite repeat (STR 2 from intron 40) were analyzed for 70 families from the Canadian Type 1 VWD Study (Table 3). Analysis of the haplotype generated confirmed that the 2 sequence variations were in phase and that the inheritance pattern of the sequence variations is dominant. Five of the 7 Canadian families positive for the 5312−19A>C transversion shared a common haplotype for all the SNPs and for the

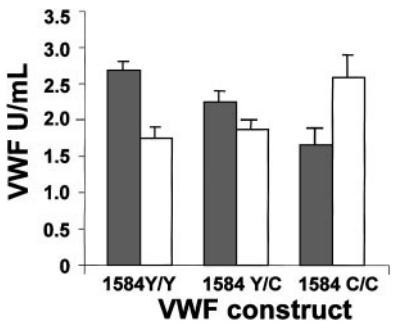

Figure 3. Expression studies demonstrating increased intracellular retention for rVWF protein containing the Tyr1584Cys mutation. Secretion of rVWF protein from COS-7 cells transfected with the pCIneoC1584 construct or cotransfected with wild-type and mutant constructs (heterozygous state) was decreased by 38% ($P = .005$) and 16% ($P = .07$), respectively, relative to the secretion of wild-type rVWF protein and the intracellular levels of rVWF were increased by 47% ($P = .04$) and 8% ($P = .6$), respectively. n = 4-6. ▨ indicates media; □, lysate. Error bars indicate SEM.

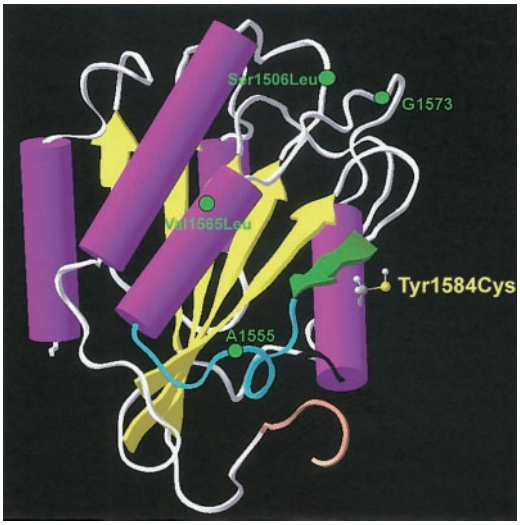

Figure 4. Homology model of VWF A2 domain showing Tyr1584Cys mutation and common polymorphisms. (A) Homology model of the VWF A2 domain was developed, based on the crystallographic structures of homologous domains from the integrins α1-β1, α2-β1, αL, and αM and from the VWF A1 and A3 domains. Tyr1584 was replaced with cysteine on alpha helix 4, and a molecular dynamic simulation was performed to assess the effect of this substitution on protein structure. No significant structural changes were observed. The reactive thiol group on the side chain of Cys1584 (indicated as a yellow ball) is 100% exposed to solvent. Common polymorphisms are also labeled in the figure.

microsatellite repeat in intron 40 (haplotype 1 = G, A, T, G, A, C, G, C, T, 6 for 2 promoter polymorphisms at nucleotides −1185 [G/A] and −1051 [A/G], exon 8, aa 318 [T/A], exon 12, aa 471 [G/A], exon 18, aa 789 [A/G], exon 28, aa 1547 [C/T], exon 28, aa 1584 [A/G], intron 30, nt −19 [A/C], exon 42, aa 2413 [C/T], and STR 2 in intron 40) (Table 3). An SNP at amino acid 484 in exon 13 was originally included in the haplotype analysis but was later excluded because this sequence variation is found at a CpG dinucleotide hypermutable element, and the phase of this SNP often could not be determined. The SNPs in the promoter of one family were not consistent with haplotype 1, an inconsistency that may be explained by a crossing-over event (haplotype 2 = A, G, T, G, A, C, G, C, T, 6). The promoter SNPs in one family are ambiguous, and it is unknown whether this family has haplotype 1 or haplotype 2. Three additional families have the 4751A>G (Tyr1584Cys) sequence variation, but not the 5312−19A>C intron change. The haplotypes in 2 of these families are the same up to the exon 28 sequence variation, but they differ at SNPs and at the microsatellite repeat located downstream of exon 28, suggesting another crossing-over event distal to this site (haplotype 3 = G, A, T, G, A, C, G, A, C, 3) (Table 3). The third family has haplotype 1 without the 5312−19A>C intron change.

The frequency of haplotypes 1, 2, and 3 in the Canadian Type 1 VWD Study families are 8.6%, 2.9%, and 2.9%, respectively, and haplotype 1 or 2 is found in 20% (2 of 10) of the families from the United Kingdom. These haplotypes have not been observed in the healthy population. This strongly suggests that the mutation in all these families is located on a "founder" type 1 VWD chromosome, and crossing-over events that occurred at the 5′ and 3′ ends of the *VWF* gene resulted in haplotypes 2 and 3 (Figure 5). Evolution of the core haplotype suggests that the mutation in these families occurred many generations ago, and the 3 different haplotypes provide evidence that the families in this study are not directly related.

Discussion

It is well recognized that the type 1 VWD phenotype shows incomplete penetrance and variable expressivity within kindreds. Recommendations concerning the diagnostic definition of type 1 VWD have been formulated, but no consensus has been reached.[31,32] Indeed, in a recent review of this problem,[33] a proposal has been made to significantly re-evaluate and revise the criteria that define the type 1 VWD phenotype. In addition to the diagnostic challenge of this disease, the molecular genetic basis of type 1 VWD has been elucidated in only a few reports, and the inheritance pattern in most of these atypical cases has been autosomal recessive.[34-37] Only 2 mutations with autosomal-dominant inheritance and high penetrance have been described in several families worldwide.[38-40] In this paper, we describe the first report of a recurring VWF mutation that is associated with a significant proportion, 14.3%, of type 1 VWD patients in a Canadian population and that has been documented in several patients in the United Kingdom. This mutation does not have complete penetrance, as is demonstrated by the patient who has the mutation but has normal VWF:Ag levels and no bleeding history.

The 5312−19A>C sequence variation in intron 30 is located at the invariant "A" in a putative intron branch site. Sequence variations in branch sites have been reported to cause aberrant splicing, such as exon skipping, and, ultimately, they can result in the synthesis of a mutant protein. However, the 5312−19A>C transversion does not result in abnormally spliced VWF products or unstable products, and both alleles of the patient's DNA are stably expressed as (platelet) RNA. Splicing is likely occurring at a different branch site in intron 30, located 27 nucleotides upstream of exon 31, because this alternative site is 100% homologous to the consensus branch site sequence, whereas the putative branch site that was investigated is only 71% identical.

The Tyr1584 residue is conserved in the VWF-A2 domain in 25 different mammalian species.[41] In addition, this residue is conserved in 3 of 6 structurally homologous domains (VWF A1 domain and the integrins α2β1 and αL), and a conservative change is found at the analogous position in the VWF A3 domain. This strong sequence conservation suggests that Tyr1584 has a structural and a functional role in VWF A–like domains. Residue 1584 is located on alpha helix 4 of our homology model, and its side chain is fully exposed to solvent. Molecular dynamic simulations did not predict any significant structural change caused by the Tyr1584Cys substitution. This suggests that the associated phenotype results from exposure of the reactive thiol group rather than from a significant structural change caused by the amino acid substitution. This reactive thiol group is capable of interacting with free thiol groups on other proteins.

The pathogenic significance of amino acid substitutions to or from cysteine residues has previously been demonstrated in numerous studies, and it is well established that unpaired cysteines may prevent the intracellular transport of many proteins.[38] Twenty-eight reported mutations in the *VWF* gene involve cysteine residues that result in different types of VWD.[10,42-45] In contrast, only 1 reported "polymorphism" in the *VWF* gene involves a substitution to cysteine (Tyr1584Cys), and we have provided evidence demonstrating that this sequence variation is a common mutation in Canadian families with type 1 VWD.

Patients with a mutation of Cys1149Arg, in exon 26, have type 1 VWD.[38] These patients have markedly reduced VWF:Ag and VWF:RCo levels, with values ranging from 0.1 to 0.25 U/mL. Cys1149 forms an intramolecular disulfide bond with Cys1169,

Table 3. Haplotype data for VWD type 1 families

Family	Relationship to propositus	Status	Haplotype chromosome 1	Haplotype chromosome 2	Founder haplotype
2-4	Propositus	Y	G, A, T, G, A, C, G, C, T, 6	G, A, T, G, A, C, A, A, C, 4	1
2-5	Son	Y	G, A, T, G, A, C, G, C, T, 6	A, G, T, G, A, C, A, A, C, 4	1
2-62	Husband	N	G, A, T, A, G, T, A, A, C, 4	A, G, T, G, A, C, A, A, C, 4	NA
2-63	Son	Y	G, A, T, G, A, C, G, C, T, 6	A, G, T, G, A, C, A, A, C, 4	1
17-43	Propositus	Y	G, A, T, G, A, C, G, C, T, 6	A, G, T, G, A, T, A, A, T, 1	1
17-44	Father	U*	G, A, T, G, A, C, G, C, T, 6	A, G, T, A, G, C, A, A, C, 4	1
17-45	Mother	N	A, G, T, G, G, T, A, A, C, 3	A, G, T, G, A, T, A, A, C, 1	NA
17-46	Sister	N	A, G, T, G, G, T, A, A, C, 3	A, G, T, A, G, C, A, A, C, 4	NA
33-107	Propositus	Y	G, A, T, G, A, C, G, C, T, 6	G, A, T, G, A, C, A, A, C, 3	1
33-108	Father	U*	G, A, T, G, A, C, G, C, T, 6	G, A, T, G, A, C, A, A, C, 5	1
33-109	Mother	U	A, G, T, A, G, C, A, A, C, 4	G, A, T, G, A, C, A, A, C, 3	NA
34-110	Propositus	Y	G, A, T, G, A, C, G, C, T, 6	G, A, T, A, G, T, A, A, C, 4	1
34-111	Mother	Y	G, A, T, G, A, C, G, C, T, 6	G, A, T, G, A, C, A, A, C, 4	1
34-112	Sister	N	G, A, T, A, G, T, A, A, C, 4	G, A, T, G, A, C, A, A, C, 4	NA
70-236	Propositus	Y	G, A, T, V, V, C, G, C, T, 6	A, G, T, V, V, C, A, C, C, 4	1
70-237	Brother	Y	G, A, T, V, V, C, G, C, T, 6	G, A, T, V, V, C, A, C, T, 6	1
70-238	Father	Y	G, A, T, V, V, C, G, C, T, 6	A, G, T, V, V, T, A, C, C, 4	1
59-196	Propositus	Y	G, A, T, G, A, C, G, A, T, 6	A, G, T, G, A, C, A, A, C, 4	1‡
59-197	Father	U	A, G, T, G, G, T, A, A, C, 4	A, G, T, G, A, C, A, A, C, 4	NA
59-198	Mother	U†	G, A, T, G, A, C, G, A, T, 6	A, G, T, G, G, T, A, A, C, 4	1‡
59-199	Sister	U†	G, A, T, G, A, C, G, A, T, 6	A, G, T, G, G, T, A, A, C, 4	1‡
66-218	Propositus	Y	A, G, T, G, A, C, G, C, V, 3/6	G, A, T, G, A, C, A, A, V, 3/6	2
66-219	Mother	N	A, G, T, G, A, C, G, C, V, 3/6	A, G, T, G, G, T, A, A, V, 3/6	2
11-22	Propositus	Y	V, V, T, G, V, V, G, C, T, 6	V, V, T, G, V, V, A, A, C, 3	1 or 2
11-23	Mother	U	V, V, T, G, V, V, A, A, C, 4	V, V, T, G, V, V, A, A, C, 3	NA
11-24	Brother	U*	V, V, T, G, V, V, G, C, T, 6	V, V, T, G, V, V, A, A, C, 3	1 or 2
31-100	Propositus	Y	G, A, T, G, A, C, G, A, C, 3/5	G, A, T, G, A, C, A, A, C, 3/5	3
31-101	Brother	N	G, A, T, G, A, C, A, A, C, 6	G, A, T, G, A, C, A, A, C, 5	NA
31-102	Brother	N	G, A, T, G, A, C, A, A, C, 6	G, A, T, G, A, C, A, A, C, 6	NA
31-103	Brother	N	G, A, T, G, A, C, A, A, C, 6	G, A, T, G, A, C, A, A, C, 6	NA
49-159	Propositus	Y	G, A, T, G, A, C, G, A, C, 3	G, A, T, G, A, C, A, A, C, 3	3
49-160	Father	Y	G, A, T, G, A, C, G, A, C, 3	A, G, T, G, G, T, A, A, C, 4	3
49-161	Mother	N	A, G, T, G, A, T, A, A, C, 1	G, A, T, G, A, C, A, A, C, 3	NA

In the left column, the first number indicates the family study number and the second number indicates the individual's study number, as recorded in our patient database. Haplotypes were derived from restriction enzyme analysis of amplified VWF and from the number of TCTA repeats found at STR 2 in intron 40. Each haplotype, in the order of appearance in the table, includes two promoter polymorphisms at nucleotides −1185 (G+/A−, *Bst* UI) and −1051 (A+/G−, *Nla*III), exon 8 aa 318 (T+/A−, *Msl* I), exon 12 aa 471 (G+/A−, *Mae*II), exon 18 aa 789 (A+/G−, *Rsa*I), exon 28 aa 1547 (C+/T−, *Bst* EII), exon 28 aa 1584 (A+/G−, *Kpn*I), intron 30 nt −19 (A−/C+, *Bsp*1286I), exon 42 aa 2413 (C+/T−, *Aci* I), and STR 2 in intron 40 (number of TCTA repeats is indicated). The common haplotype segregating with affected patients is underlined. V indicates that data at that position were ambiguous. Y indicates affected status; N, unaffected; U, unknown; and NA, not applicable.

*Individual has no reported bleeding history and is not available for clinical testing.

†Individual is undiagnosed but has prolonged in vitro bleeding time.

‡Haplotype 1 without the 5312−19A>C sequence variation.

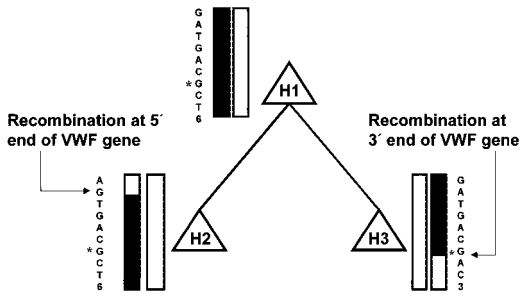

Figure 5. Evolution of the common VWF haplotype associated with type 1 VWD. This schematic illustrates the proposed evolution of the common VWF haplotype. The original VWF DNA had a common core haplotype (H1) of GATGACGCT6 (haplotype includes 2 promoter polymorphisms at nucleotides −1185 [G+/A−, *Bst* UI] and −1051 [A+/G−, *Nla*III]; exon 8, aa 318 [T+/A−, *Msl* I]; exon 12, aa 471 [G+/A−, *Aat* II]; exon 18, aa 789 [A+/G−, *Rsa*I]; exon 28, aa 1547 [C+/T−, *Bst* EII]; exon 28, aa 1584 [A+/G−, *Kpn*I] intron 30, nt −19 [A−/C+, *Bsp*1286I]; exon 42, aa 2413 [C+/T−, *Aci* I]; and STR 2 in intron 40. The site of the Tyr1584Cys mutation is indicated with an asterisk. At some point in history, a spontaneous mutation of G>A occurred at the CpG dinucleotide in exon 13. This was followed later by 2 crossing-over events in the *VWF* gene—one at the 5′ end of the gene (H2, AGTGACGCT6) and the other between exon 28 and intron 30 (H3, GATGACGAC3). Black bars indicate the affected chromosome and white bars indicate the unaffected chromosome. In H2 and H3, the wild-type chromosome has crossed onto the affected chromosome.

and expression studies have demonstrated that the unpaired thiol group of Cys1169 does not account for the intracellular retention of Cys1149Arg. It is unknown whether Cys1149 and Cys1169 are surface exposed; however, a conformational change in protein structure is expected because a stabilizing disulfide bond has been broken. The molecular mechanism of the Cys1149 mutation has been recently determined.[38] Homodimer and heterodimer formation occurs in the endoplasmic reticulum, following the synthesis of VWF protein. This dimerization process is random, and approximately 75% of dimers contain a mutant subunit. These mutant dimers are retained in the endoplasmic reticulum, and approximately 25% of the normal homodimers are transported to the Golgi complex for assembly into multimers. These numbers are consistent with the VWF:Ag levels observed in patients. A similar mechanism may explain the decreased VWF:Ag levels observed in patients with the Arg1374Cys mutation.

The dominant-negative mechanism described does not explain the decreased levels of VWF in patients with the Tyr1584Cys mutation because these patients have VWF:Ag levels of approximately 0.40 U/mL rather than approximately

0.25 U/mL. VWF:Ag levels of approximately 0.40 U/mL could suggest heterozygosity for a null allele (ie, a haploinsufficiency mechanism); however, one patient described in this study (Family I-2) is homozygous for the Tyr1584Cys mutation but still has a detectable VWF:Ag level of 0.24 U/mL rather than 0 U/mL, which would be expected for the inheritance of 2 null alleles. The VWF:Ag level for the homozygous Cys1584 patient is significantly lower than in heterozygotes with this mutation, suggesting that the inheritance of this mutation is codominant. This in turn suggests that the pathogenic mechanism of the Tyr1584Cys mutation is more likely to involve an inherent instability of the mutant protein because of its reactive free thiol group interacting with VWF or with another protein, resulting in VWF protein degradation during its intracellular biosynthesis or in accelerated clearance from the circulation. In accordance with this, expression studies using recombinant Tyr1584Cys VWF protein have revealed that the Tyr1584Cys mutation results in increased intracellular retention. These results suggest that the low plasma levels of VWF in these patients are the consequence, at least in part, of reduced secretion of protein, though other extracellular mechanisms may also be contributing factors.

We propose that a common founder for the 4751A>G mutation has been indicated in this study by the presence of a common core VWF haplotype. This haplotype was found in patients from across Canada and in several patients from Southampton, United Kingdom, and the fact that 5312−19A>C was originally identified in France suggests that this genetic sequence is common in type 1 VWD patients from multiple ethnicities. The occurrence of an evolved haplotype provides support that the mutation in the patients from this study is ancient. The mutation segregates with the affected members in each family, demonstrating a codominant mode of inheritance; this is especially obvious in family 1, in whom all the children of the homozygous father (4751A>G) are affected with type 1 VWD. Several of the members with this genetic sequence remain undiagnosed and are unavailable for testing or refused diagnostic testing (33-108, 11-24, 17-44) or are reported to be unaffected (66-219). Most of these persons are male, emphasizing the fact that women are more likely to report excessive mucocutaneous bleeding symptoms because of menorrhagia.[46] The undiagnosed or reportedly unaffected family members in this study might have never had a significant hemostatic challenge that required medical attention or they may have coinherited traits that nullify the manifestation of VWD (eg, ABO blood type).

In conclusion, this is the first study to describe a VWF mutation that is associated with a significant proportion of type 1 VWD patients from several countries. This mutation has been detected in families that have a clear autosomal codominant pattern of inheritance. Molecular genetic testing for the Tyr1584Cys mutation (4751A>G) is straightforward and may now be incorporated into the diagnostic assessment of patients who are being investigated for type 1 VWD.

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