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ORIGINAL ARTICLE

Search for Mutations in a Segment of the Exon 28 of the Human von Willebrand Factor Gene: New Mutations, R1315C and R1341W, Associated With Type 2M and 2B Variants

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Abstract

von Willebrand Disease (vWD) is the most frequently inherited bleeding disorder in humans, and is caused by a qualitative and/or quantitative abnormality of the von Willebrand factor (vWF). A large number of defects that cause qualitative variants have been located in the A1 domain of the vWF, which contains sites for interaction with platelet glycoprotein Ib (GPIb). We have developed a new approach to detect mutations based on DdeI digestion and single-strand conformation polymorphism analysis. A segment of 487 nucleotides, extending from intron 27 to codon 1368 of the pre-pro vWF was amplified from genomic DNA. The cleavage with DdeI yields two fragments of appropriate size for this kind of analysis and confirms that the gene, rather than the pseudogene, is being investigated. Six families with type 2B vWD, one type 2M vWD family, and one another type 2A vWD family were studied. After sequencing the fragments with an altered electrophoretic pattern, we found four mutations previously described—R1308C, V1316M, P1337L, and R1306W—in patients with 2B vWD. The last one arose de novo in the patient. In addition, two new candidate mutations were observed: R1315C and R1341W. The first one was associated to type 2M vWD, whereas the one second cosegregated with type 2B vWD. The fact that these new mutations were not found in 100 normal alleles screened further supports their causal relationship with the disease. These mutations, which induce either a gain or a loss of function, further show an important regulatory role of this region in the binding of vWF to GPIb and its implications in causing disease.

Keywords: von Willebrand Disease; von Willebrand factor gene; A1 domain vWF; single strand conformation polymorphism; type 2 vWD

INTRODUCTION

von Willebrand Disease (vWD) is an autosomally inherited bleeding disorder caused by a qualitative and/or quantitative abnormality of von Willebrand factor (vWF) [1]. Human vWF is a large multimeric glycoprotein that plays an essential role in the adhesion of platelets to the subendothelium under the high shear stress that is prevalent in the microcirculation of the blood. It also acts as a carrier for coagulation factor VIII [2]. The vWF cDNA was cloned by several groups simultaneously between 1985 and 1986. The vWF gene, located on the short arm of chromosome 12 [3,4] spans 178 kb in length and contains 52 exons [5]. There is also a pseudogene on chromosome 22 [6] spanning exons 23–34 of the vWF gene. These sequences have about 97% homology, which makes it difficult to detect mutations in these regions. vWF is synthesized in the megakaryocytes and endothelial cells from an 8.8 kb transcript of the gene that encodes a precursor protein of 2813 amino acids (aa), which contains four different repeated domains. The pre-pro vWF includes a typical 22 aa signal peptide, a pro-peptide of 741 aa and the mature subunit of 2050 aa that contains all functional domains. After complex post-translational modifications [7] the protein is stored in the a-granules of the platelets and in the Weibel-Palade body of the endothelial cells. In spite of the heterogeneous nature of vWD, it is caused by defects at the vWF locus. The revised classification [8] of vWD distinguishes between partial quantitative (type 1), qualitative (type 2), and total quantitative (type 3) deficiencies of vWF. Qualitative defects are divided in four subcategories—2A, 2B, 2M, and 2N. Type 2A is a qualitative variant characterized by a reduced platelet-dependent function, associated with an absence of large multimers, while type 2M vWD refers to variants with decreased platelet-dependent function that are not caused by the absence of large multimers. Type 2B vWD refers to variants with increased affinity for platelet glycoprotein Ib (GPIb), and

finally type 2N refers to variants with decreased affinity for factor VIII. When all types are considered, vWD is the most common inherit ed human bleeding disorder. Approximately one out of every 8,000 individuals have symptomatic vWD, whereas asymptomatic inherited defects in vWF are detectable in nearly 1% of the population [9]. The binding domain to the platelet GPIb was located in a fragment of the mature subunit (aa 449–728) [10] that includes the A1 domain of vWF. A large number of missense mutations resulting in vWD type 2B have been located in this domain [11] (vWF database, http://mmg2.im.med.umich.edu/vWF/), the majority of them are confined to a short peptide that extends from amino acid 540 to 580.

The present report describes the search for mutations in a fragment of 487 bp (base pairs) that spans from intron 27 to codon 605 of the mature subunit of the vWF. Patients that showed an increased or decreased affinity for GPIb were studied, and the analysis was performed in families previously classified as type 2B, 2M, or 2A, and in their relatives. Type 1 vWD patients with a slight discrepancy between the antigen and the function of vWF, and unclassified vWD patients were also included in order to test the possibility that these patients could have a genetic alteration in this functional domain. Two new candidate mutations, the R1341W and the R1315C were found in two families with type 2B and 2M, respectively.

MATERIAL AND METHODS

Patients

The study involved 17 type 2B vWD patients from six families, one type 2A patient in which any mutation was found in the A2 domain, 2 type 2M patients from the same family, and 15 patients from seven families with type 1 vWD or unclassified. The latter patients were selected because they showed a slight decrease in vWF: RCo vs. vWF:Ag.

Thirty-nine unaffected family members were also studied. All families were aware of the investigative nature of the studies, and gave their consent.

Functional and Antigenic Assays

The bleeding time was measured by Ivy method using Simplate II. The plasma samples were obtained from blood collected with 0.129 M sodium citrate (9:1). The factor VIII:C was measured by the *one-stage* assay. The ristocetin cofactor (vWF:RCo) was determined using formalin-fixed platelets as previously described [12]. The antigen (vWF:Ag) was determined by the enzyme-linked immunosorbent assay (ELISA) method [13]. Ristocetininduced platelet aggregation (RIPA) was measured in platelet rich plasma (PRP) using an Aggrecorder II aggregometer (Kyoto Daiichi Kagaku Co., Japan). The function of plasmatic vWF in platelet poor plasma (PPP) vs. washed normal platelets was analyzed by RIPA [14].

Multimeric assays

The multimeric structure analysis was performed according to the original method described by Ruggeri and Zimmerman [15]. In summary, the non-reduced samples were analyzed by sodium dodecyl sulfate (SDS)-agarose electrophoresis (1.4% and 2.2%). The multimers were identified using peroxidase labelled antibodies and detected by luminol after blotting to an immobilon membrane [16].

DNA Extraction

The DNA was extracted from blood collected in ethylene diaminetetraacetic acid (EDTA) using a standard method [17].

Polymorphisms Segregation Analysis

The following microsatellites were analyzed: VNTR3, VNTR1, and VNTR2 (all of them located in intron 40 of the vWF gene, corresponding to 1640–1794, 1890–1991, and 2215–2380 nucleotides, respectively) [18], and another one in the promoter region (1490–1665 nucleotides). The *Rsa*I polymorphism located on exon 18 (15/292 nucleotide), and the *BstE*I on exon 28 (24/1172 nucleotide) were also analyzed. The nucleotide numbering used is according to Mancuso et al. [5].

PCR Amplification

A 487 bp fragment containing part of exon 28 of the vWF gene, and extending from intron 27 to codon 1368 was amplified by polymerase chain reaction (PCR) using primers A (58-AGAAGTGTCCACAGGTTCTTC-38) and B (58-AGATTTGGAACAGTGTGTATTTCAAGACCT-38), nucleotides 7560–8046 [6]. These primers were specific for the gene sequence. The reaction volume was 50 ml, containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl2, and 0.1% Triton X-100 (pH 8.8), 1U of DNA polymerase (DynaZyme™, Finnzymes Oy, Finland), 50 mM of each dNTP, 200 ng of genomic DNA, and 0.25 mM of each primer. The PCR conditions were initial denaturation at 94°C for five min followed by 30 cycles of 94°C for one min, 55°C for one min, 72°C for two min and a final step of 72°C for seven min.

Single-Strand Conformation Polymorphism Analysis (SSCP)

The product of PCR was diluted three times and digested with *Dde*I. A volume of loading buffer was then added. The samples were heated to 95°C for five min and then cooled in ice. Five ml of each mixture was applied to 10% polyacrylamide (29:1) gels and run for 16 hr at 10 watts. The bands were detected by staining with silver nitrate.

Mutation's detection

The amplified DNA was purified using microfiltration (Centricon-100), and quantified by electrophoresis vs. φX174. After that, 30 ng of DNA were sequenced by the fluorescent dideoxy terminator method and analyzed in an ABIPRISM DNA sequencer with the A and B primers. To detect the C4193T (R1315C) change (family AS), a 189 bp fragment was amplified with the B and C primers (C: 58-GGCTGCGCATCTCCCAGAAGTaGaTC-38, where the modified nucleotides are written with underlined small letters). When the mutation was present, the primer C created a *Bgl*II restriction site. The PCR conditions were the same as those described above, except that 1.5 mM MgCl2 was used, and the annealing temperature was 65°C. The R1341C substitution was detected by *Ita*I restriction analysis of the same fragment. The different bands were analyzed in 12% and 15% polyacrylamide minigels, and silver stained.

Sequence Analysis

The sequence analysis was performed with the GCG program from Wisconsin Sequence Analysis Package.

RESULTS

The amplification from genomic DNA with primers A and B yielded the expected 487 bp fragment, which includes 57 bp from intron 27 and 430 bp from exon 28, until nucleotide 4355 of the cDNA (codon 1368 pre-pro vWF). The nucleotides of the cDNA sequences are numbered assigning +1 to the major transcription cap, which is 250 nucleotides upstream the initiation codon for methionine [19]. The fragment amplified was specific for the gene and the cleavage with *Dde*I yielded two bands of 254 and 233

bp. The corresponding fragment in the pseudogene would have given three bands of 168, 86, and 233 bp. The SSCP analysis showed different electrophoretic mobilities in samples corresponding to patients. One sample per family was sequenced with primers A and B. This allowed us to detect four previously described mutations (vWF database). The R1308C, V1316M, and P1337L mutations were found in patients from three families with a typical 2B phenotype. By restriction analysis, it was confirmed that these mutations segregated with the disease in each family. The R1306W substitution was found in a sporadic patient who also had a classical 2B phenotype. The SSCP analysis in the parents and seven brothers verified that the mutation arose de novo in the patient. In all cases, the patients were heterozygous for the mutations. We also could detect two new candidate mutations.

R1315C Mutation

This substitution was detected in individual I:1 from family AS. Laboratory data on clinical material are shown in Table I. It is worth noting that patient I:1 had vWF:RCo/vWF:Ag ratios of less than 0.3, and both I:1 and II:2 had less than 6% of vWF:RCo in more than three measurements. On the other hand, they repeatedly showed all the vWF multimers in plasma (Fig. 1). Both of these patients have a moderate to severe diathesis and it was necessary to resort to plasma concentrates containing factor VIII and vWF in many instances. Both the family structure and the polymorphism segregation analysis suggest that vWD segregated with the locus of the vWF gene in one autosomal dominant pattern (Fig. 2). The SSCP analysis showed an altered electrophoretic pattern in samples from both patients, I:1 and II:2 (Fig. 3). The posterior sequencing of the purified DNA from I:1 made it possible to identify the transition C4193T as a heterozygous mutation. This produces the R1315C substitution in the pre-

pro vWF that corresponds with Arg552Cys of the mature subunit. The amplification from genomic DNA with primer B and the modified primer C yielded a 189 bp fragment. The cleavage with *Bgl*II produced two bands of 167 bp and 22 bp. As can be seen in Figure 4B, lanes 4 and 5 (II:1 and I:1 samples), the 189 bp band corresponds to the normal allele and another of 168 bp corresponds to the mutated allele (the small 22 bp band was not present in the gel). This kind of analysis was performed in 100 unrelated normal alleles, and this transition was not detected.

R1341W Mutation

Individuals III:2, III:4, and II:2 exhibited a 2B vWD phenotype (Table I), showed an increased ristocetin sensitivity, and all the vWF multimers were present in their plasma. In addition, they had a mild bleeding diathesis. The analysis with intragenic markers was concordant with a defect at the vWF gene locus inherited in a autosomal dominant fashion. The SSCP analysis showed an altered electrophoretic pattern in the samples from III:2, III:4, and II:2. By automated sequencing, we detected a thymidine (T) in addition to the normal cytidine (C) at nucleotide 4271 in individual III:2. This C4271T change, which produced the anomalous migration of the bands in SSCP analysis, led the 1341 codon from pre-pro vWF to encode a tryptophan instead of the normal arginine that corresponds to Arg578Trp in the mature subunit. This candidate mutation has not been described previously, and we detected it in the other patients because the transition C4271T destroys two restriction sites of the *Ita*I enzyme. The 189 bp fragment obtained with primers B and C produced fragments of three bp (two), 99 bp, 32 bp, and 52 bp in the normal alleles, whereas the mutation yielded segments of 3 bp, 134 bp, and 52 bp, as can be seen in Figure 4C. This change was excluded from the rest of the family

members analyzed. A search of this mutation in 100 unrelated normal alleles was negative.

No altered electrophoretic pattern was found by SSCP analysis in the remaining family with a 2B vWD phenotype. No mutation could be identified by later sequencing of the 487 bp genomic fragment. Moreover, an altered pattern SSCP analysis was not observed in the type 2A sporadic patient, neither in the remaining type 1 vWD or unclassified families analyzed. Studies in other regions are currently in progress.

DISCUSSION

We have developed a method for searching for mutations in a segment that includes part of the domain A1 and a short part of the domain D3, based on PCR amplification, *Dde*I digestion, and SSCP analysis. The *Dde*I digestion has a double use: It generates fragments of an appropriate size for SSCP analysis, and confirms that the gene, and not the pseudogene, is being investigated. In this way, we have identified the mutation causing vWD in five out of the six families with type 2B vWD, and in the 2M phenotype family studied.

The R1308C, V1316M, R1306W, and P1337L mutations that we found in four families with a typical 2B vWD phenotype have been described multiple times (database). Some patients showed thrombocytopenia several times, especially in stressful situations or in others that are known to increase the vWF plasma concentration and aggravate the haemostatic abnormalities in 2B patients, but on the whole they had moderate symptoms. The loss of high molecular weight (HMW) plasma multimers was more or less pronounced [20]. Several functional studies have been performed, and in one of the

latest, R1308C, V1316M, and R1306W substitutions were analyzed in the same system assay. This study demonstrated that these mutations produced similar binding of the vWF to platelets both spontaneously and when induced by ristocetin [21].

We found the transition C4193T that produces the R1315C mutant protein in a patient with 2M vWD [22]. The same mutation was simultaneously reported in patients classified as 2A. Nevertheless, the clinical and laboratory characteristics of patients with this variant have not been detailed. This disagreement appears similar to two other cases with a 2A phenotype that share the same mutations proven to cause 2B vWD [23]. Our patients had a moderate-to-severe bleeding diathesis, the laboratory data were compatible with a qualitative 2M variant with lower vWF:RCo/vWF:Ag ratios, and all of the vWF multimers were detected in plasma in repetitive testing. The real reason for this discrepancy is still not clear. A second mutation elsewhere in the same gene may be implicated in the differences observed. However, variants in other loci outside the vWF gene could also be involved. Platelet vWF studies and functional assays with recombinant protein may help elucidate this discrepancy between phenotype and genotype. In any case, the substitution of the arginine, positively charged under physiological conditions by cysteine, which is able to establish a disulphide bond, is important enough to be considered the cause of the disease.

The R1341W mutant protein produced by the transition C4271T was detected in three patients of the SLL family. They showed a type 2B vWD phenotype characterized by increased ristocetin sensitivity and the presence of all vWF multimers in plasma. They also had mild bleeding diathesis. The substitution of a positively charged amino acid (Arg) by another one with a big aromatic group (Trp) is a good candidate to produce a conformational change that could affect the binding of the vWF to the platelet GPIb. Both arginine at codon 1315 and 1341 are conserved in the porcine vWF gene [24], and

this may mean that they play an important role in protein function. Different substitutions of amino acids at this codon have been described (database). The R1341Q (G4272A) substitution is particularly frequent and there are functional studies that reproduce the 2B phenotype. R1341P (G4272C) have also been described in a patient with 2B vWD.

A possible change in the VNTR3 allele was observed in an SLL family. Regrettably, the parents were not available for the study. However, the analysis of different microsatellite markers at the X chromosome (data not shown) could not exclude false paternity.

Strikingly, all sequence changes found are C®T transitions within CG dinucleotides. The cytosine residue is frequently methylated [25] and may undergo deamination to yield thymine at CG dinucleotides. Such CG dinucleotides are mutational hotspots in many genes.

The loss of function can be inherited in a dominant negative fashion because both normal and defective subunits are present after multimerization. The mutations detected in the A1 domain, that induce either a gain or a loss of function, further underlines the important regulatory role of that region in the binding of vWF to GPIb, perhaps through conformational changes. On the other hand, these substitutions reaffirm the phenotypic and genetic variability in these qualitative variants.

The clinical benefits of these analyses include confirmation of clinical diagnosis, early diagnosis of asymptomatic or presymptomatic carriers, and more accurate genetic counselling that can be offered both to patients and healthy members of the family. This is best illustrated in the case in which the mutation R1306W arose de novo.

REFERENCES

1. Ruggeri ZM, Zimmerman TS: von Willebrand factor and von Willebrand disease. Blood 70:895–904, 1987.

2. Eikenboom JCJ, Reitsma PH, Brie¨t E: The inheritance and molecular genetics of von Willebrand's disease. Haemophilia 1:77–90, 1995.

3. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SH: Human von Willebrand factor (vWF): Isolation of complementary DNA (cDNA) clones and chromosomal localization. Science 228:1401–1406, 1985.

4. Verweij CL, de Vries CJM, Distel B, van Zonneveld A-J, von Kessel AG, van Mourik JA, Pannekoek H: Construction of cDNA coding for human von Willebrand factor using antibody probes for colony screening and mapping of the chromosomal gene. Nucleic Acids Res 13:4699–4717, 1985.

5. Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton-Inloes BB, Sorace JM, Alevy YG, Sadler JE: Structure of the gene for human von Willebrand factor. J Biol Chem 264:19514–19527, 1989.

6. Mancuso DJ, Tuley EA, Westfield LA, Lester-Mancuso TL, Le Beau MM, Sorace JM, Sadler JE: Human von Willebrand factor gene and pseudogene: Structural analysis and differentiation by polymerase chain reaction. Biochemistry 30:253–269, 1991.

7. Tuddenham, EGD and Cooper DN: The von Willebrand Factor and von Willebrand disease. In Fraser Roberts JA, Carter CO, eds. The Molecular Genetics of Haemostasis and its Inherited Disorders. New York: Oxford University Press, 1994, pp 374–401.

8. Sadler JE: A revised classification of von Willebrand disease. Thromb Haemost 71:520–525, 1994.

9. Sadler JE, Gralnick HR: A new classification for von Willebrand disease—Commentary. Blood 84:676–679, 1994.

10. Fujimura Y, Titani K, Holland LZ, Russell SR, Roberts JR, Elder JH, Ruggeri ZM, Zimmerman TS: von Willebrand factor. A reduced and alkylated 52/48 kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. J Biol Chem 261:381–385, 1986.

11. Ginsburg D, Sadler JE: von Willebrand disease: A database of point mutations, insertions, and deletions. Thromb Haemost 69:177–184, 1993.

12. MacFarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J: A method for assaying von Willebrand factor (ristocetin cofactor). Thromb Diath Haemorrh 34:306–308, 1975.

13. Jorquera JI, Montoro JM, Aznar JA, Perales MA, Lerma MA, Curats R, Bañuls E, Casaña P: Metodo ELISA en microplaca desarrollado con antisueros comerciales para medir el antígeno del factor von Willebrand. Sangre 34:147–150, 1989.

14. Holmberg L, Berntorp E, Donner M, Nilsson IM: von Willebrand's disease characterized by increased ristocetin sensitivity and the presence of all von Willebrand factor multimers in plasma. Blood 68:668–672, 1986.

15. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of Factor VIII/von Willebrand factor. Blood 57:1140–1143, 1981.

16. Schneppenheim R, Budde U, Dahlmann N, Rautenberg P: Luminography—a new, highly sensitive visualization method for electrophoresis. Electrophoresis 12:367–372, 1991.

17. Blin N, Stafford DW: A general method for isolation of high molecular weight DNA from eukaryocytes. Nucleic Acid Res 3:2303–2308, 1976.

18. Casaña P, Martinez F, Aznar JA, Lorenzo JI, Jorquera JI: Practical application of three polymorphic microsatellites in intron 40 of the human von Willebrand factor gene. Haemostasis 25(6):264–271, 1995.

19. Bonthron D, Orkin SH: The human von Willebrand factor gene: Structure of the 58 region. Eur J Biochem 171:51–57, 1988.

20. Casaña P, Haya S, Lorenzo JI, Martıínez F, Montoro JM, Curats R, Aznar JA: Detection of mutations and clinical manifestations in four Spanish families with type 2B von Willebrand disease. Rev Iberoam Thromb Hemost 10:15–22, 1997.

21. Cooney KA, Ginsburg D: Comparative analysis of type 2b von Willebrand disease mutations: Implications for the mechanism of von Willebrand factor binding to platelets. Blood 87:2322–2328, 1996.

22. Casaña P, Lorenzo JI, Martínez F, Haya S, Montoro JM, Aznar JA: Single-strand conformation polymorphism analysis in the A1 domain of the von Willebrand factor gene (Abstract). Thromb Haemostasis (Suppl.), June 1997:655–656, 1997.

23. Meyer D, Fressinaud E, Gaucher C, Lavergne JM, Hilbert L, Ribba AS, Jorieux S, Mazurier C: Gene defects in 150 unrelated French cases with type 2 von Willebrand disease: From the patient to the gene. Thromb Haemost 78:451–456, 1997.

24. Bahnak BR, Lavergne JM, Ferreira V, Kerbiriou-Nabias D, Meyer D: Comparison of the primary structure of the functional domains of human and porcine von Willebrand factor that mediate platelet adhesion. Biochem Biophys Res Commun 182:561–568, 1992.

25. Bird AP: DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504, 1980.