RAPID COMMUNICATION

Characterization of von Willebrand Factor Gene Defects in Two Unrelated Patients With Type IIC von Willebrand Disease

By Christine Gaucher, Jocelyne Diéval, and Claudine Mazurier

Genetic studies were performed in two unrelated patients with the IIC phenotype of von Willebrand disease (vWD) characterized by the increased concentration of the protomeric form of von Willebrand factor (vWF). In patient B, the sequencing of both exons 15 and 16 of the vWF gene showed two sequence alterations: a 3-bp insertion in exon 15 resulting in the insertion of a Glycine at position 625 (625insGly) and a 2-bp deletion in exon 16 leading to a premature translational stop at codon 711 (711 ter), at the heterozygote state. Patient A was found homozygous for a single point mutation also localized in exon 15 and responsible for the substitution Cys623Trp. These candidate mutations were not found in a panel of 96 normal chromosomes, suggesting a causal relationship with IIC vWD phenotypic expression. The composite heterozygote or homozygote state of both patients supports the recessive mode of inheritance already described for this phenotype. Furthermore, the localization of these gene defects in the D2 domain of vWF propeptide, known to play an important role in vWF multimerization, provides another argument in favor of their causative effect regarding the peculiar multimeric pattern of vWF in these patients.

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vWF GENE DEFECTS IN IIC vWD PHENOTYPE

Fig 1. Pedigree and genetic study of family B. The propositus (II14) is represented with a solid symbol. The members characterized with the vWF gene defect (625insGly) associated with a CIE double-peak pattern of plasma vWF are shown with half-solid symbols whereas those exhibiting the quantitative vWF gene defect (711ter) are identified by half-hatched symbols. For members II2 and II5, no DNA sample was available, but the phenotypic analysis has shown quantitative and qualitative defect of vWF, respectively. The capital letters referring to the alleles for the VNTR polymorphism localized in the intron 40 of vWF gene have been already described, except the allele R, which corresponds to the association of the 33C, 276-, and 244-bp Alu I fragments.

We report here the first characterization, in two independent families, of gene defects inducing aa changes in the D2 domain of vWF propeptide potentially responsible for vWD IIC phenotype. One patient of consanguineous origin is homozygous for the substitution Cys623Trp. The second family patient, whose phenotype has been previously described, is a composite heterozygote with the paternal allele presenting a translational stop at position 711 and the maternal allele harboring a 3-bp insertion resulting in an additional Gly residue at position 625 (625insGly). These findings confirm the recessive mode of transmission of the IIC category in type 2A vWD and highlight the importance of the D2 domain of the vWF propeptide in the multimer assembly.

MATERIALS AND METHODS

Patients

Patient A was diagnosed when she was 64 years old, although she had presented life-long bleeding symptoms. During childhood, she had epistaxis and hematemesis, and then presented menorrhagia and severe hemorrhages occurring with two spontaneous abortions. She has received transfusions on many occasions. One of her sisters died at 3 years of age from tonsillitis-induced hemorrhage. Nevertheless, her parents, who were first cousins, and her two sons had or have no bleeding symptoms.

The phenotypic data of patient B and of 11 of her family members have been already described. The family tree reported in Fig 1 summarizes the study of the vWF gene performed in this family.

Phenotype Analysis

All the laboratory tests performed have been previously described. Plasma vWF multimeric pattern was studied by electrophoresis in sodium dodecyl sulfate (SDS) 2.5% agarose, as previously described. The percentage of the different multimeric forms was measured by densitometric scanning.

DNA Sequence Analysis

Sequencing of polymerase chain reaction (PCR)-amplified DNA. Genomic DNA was purified from peripheral white blood cells on an automated nucleic acids extractor (Applied Biosystems, Foster City, CA). Several regions of the vWF gene were amplified using as primers oligonucleotides localized in adjacent intron sequences derived from the gene sequence data already published. The use of 5'-biotinylated PCR primers and the purification of single-stranded DNA on streptavidin-coated beads (Dynal, Oslo, Norway) according to the manufacturer's instructions allowed subsequent direct solid-phase sequencing of each amplified exon using T7 DNA polymerase (Pharmacia, Uppsala, Sweden). Exon 15 was amplified using primers 103 and 28 (Table 1) and 1 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in a 100 μL final reaction mixture and 35 cycles of 2 minutes at 98°C and 4 minutes at 72°C, according to the procedure recently described for the amplification of GC-rich sequences. The PCR amplification of exon 16 was performed using primers 59 and 60 (Table 1) under standard conditions: 30 cycles of 1 minute at 95°C, 1 minute at 55°C, and 2 minutes at 72°C. Subsequent sequencing reactions were performed with either the PCR primers already reported or internal oligonucleotides: primers 57, 107, and 128 for exon 15; and 165 for exon 16 (Table 1).

Study of vWF intron 40 VNTR polymorphism. VNTR analysis was performed as already described except for the primer set. Primer 31b, designed 91 bp upstream of the original primer 31, was used for amplification together with the original antisense primer 32. This change provides more informativity, with precise identification of each Alu I fragment position within the VNTR sequence.
in 45 normal individuals, whereas patient had normal bleeding time as well as FVIII and vWF levels.

Patient A, like the previously described patient B, have vWF level lower than the normal range established in VNTR sequence amplification.

**Phenotype Analysis**

In Table 1 are reported the laboratory data of both patients of similar age and sex and who were available for the preliminary study. It is noteworthy that these two asymptomatic individuals, who are the paternal grandmother and aunt of patient A, were not available for the preliminary study. It is noteworthy that these two asymptomatic individuals, who are the paternal grandmother and aunt of patient A, who were not available for the preliminary study.

**RESULTS**

In Table 2 are reported the laboratory data of both patients and of 2 additional members (II2 and IIIbis in Fig 1) of patient B’s family who were not available for the preliminary study. It is noteworthy that these two asymptomatic individuals, who are the paternal grandmother and aunt of patient B, have vWF level lower than the normal range established in 45 normal individuals, whereas patient B’s father (II1) had normal bleeding time as well as FVIII and vWF levels.

**DNA Sequence Analysis**

In patient B, several vWF gene regions were sequenced in an attempt to identify mutations that may be related to type IIC vWD: nt -37 to 1171 containing exons 2 to 10, nt 1432 to 1728 corresponding to exons 13 and 14, nt 2187 to 2527 containing exons 17 to 29, nt 5292 to 6769 containing exons 17 to 29, nt 5292 to 6769, and nt 7888 to 8476 including exons 40 to 52 (nucleotides being numbered according to the vWF cDNA sequence assigned to the ‘‘A’’ of the initiation codon).

**Table 1. Oligonucleotide Primers Sequences**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
<td>5’-AGGGAGTGGTACCCGGACGGTTG-3’</td>
<td>Intron 14 (+)</td>
</tr>
<tr>
<td>29</td>
<td>5’-CCCCCAGGGCTGCCCCCGACGG-3’</td>
<td>Intron 14 (-)</td>
</tr>
<tr>
<td>59</td>
<td>5’-ACCCACTCTCCTCTCTTCCAG-3’</td>
<td>Intron 16 (+)</td>
</tr>
<tr>
<td>60</td>
<td>5’-TGGTTTTAGGTTCTCGTACAC-3’</td>
<td>Intron 16 (-)</td>
</tr>
<tr>
<td>57</td>
<td>5’-CCCCCCTCCTCCACACCC-3’</td>
<td>Intron 14 (+)</td>
</tr>
<tr>
<td>107</td>
<td>5’-GTGCCCTAGCGGCTGCT-3’</td>
<td>Exon 15 (nt1789-1806) (+)</td>
</tr>
<tr>
<td>128</td>
<td>5’-GAACCGACACAGCGCCCGCTGC-3’</td>
<td>Exon 15 (nt1930-1944) and intron 16(+)</td>
</tr>
<tr>
<td>165</td>
<td>5’-GGAATGCAATGAGGCTGGTG-3’</td>
<td>Exon 16 (nt2031-2046) (+)</td>
</tr>
</tbody>
</table>

The oligonucleotides marked with a (+) are coding strand, whereas the ones marked (-) are antisense. Nucleotides are numbered according to the sequence of vWF cDNA, assigning nucleotide 1 to the ‘‘A’’ of initiation codon.

trofery patterns obtained in 2.5% agarose gel (Fig 2) displayed in both patients the predominance of the protomer (fastest moving band) and the absence of both HMW multimers and satellite bands that are particular features of subtype IIC vWD.

**Table 2. Laboratory Data**

<table>
<thead>
<tr>
<th>Individual and Relationship to the Propositus</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bleeding Time</td>
</tr>
<tr>
<td>Patient A*</td>
<td>&gt;30</td>
</tr>
<tr>
<td>III4* Patient B</td>
<td>&gt;20</td>
</tr>
<tr>
<td>I21  Paternal grandmother</td>
<td>—</td>
</tr>
<tr>
<td>III1bis* Paternal aunt</td>
<td>—</td>
</tr>
<tr>
<td>Controls (n = 45)</td>
<td>&lt;9</td>
</tr>
</tbody>
</table>

* Mean of three determinations.
† Studied only once.

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zygous for the insertion as well as her twin brother (III3). The sequencing of the parents DNA showed that both III3 and III4 inherited the defect from their mother (II2). The study of the adjacent exon 16 in patient B also showed a 2-bp deletion CT (2124-2125) at codons 708-709 (Fig 4) that was confirmed by sequencing the opposite strand using oligonucleotide 60. This deletion disrupts the reading frame and results in an early translational stop codon at position 711 of the propeptide (711 ter). The patient was heterozygous for this mutation. She inherited this defect from her father (III1), who was a heterozygote for the deletion (Fig 4), as was his stepsister (IIIbis). The segregation of the intron 40 VNTR alleles in family B is shown in Fig 1. The 711ter mutation is linked to the allele N, which was found in patient B (III4), her father (III1), and her paternal aunt (IIIbis), whereas the Gly625 insertion is associated with allele F, which is carried by the patient (III4), her twin brother (III3), her mother (II2), and one of her maternal aunts (II4).

In patient A, a C to G transversion (C1869G) was identified in exon 15 that results in the substitution of tryptophane for Cysteine at position 623 of the vWF propeptide (Fig 3B). The propositus was found homozygous for the defect.

**SSCP Screening**

To verify that the possible mutations identified in exon 15 were not common polymorphisms, the screening of 96 normal alleles was performed using the SSCP detection approach. The electrophoretic pattern observed for this exon in either patient A (upper gel part) or B (lower gel part) could not be found in any of the 96 normal alleles screened (Fig 5), indicating that the two sequence changes observed do not represent at least common polymorphisms.

**DISCUSSION**

In the recently published revised classification of vWD, type 2A vWD refers to qualitative variants with decreased platelet-dependent function associated with the absence of HMW vWF multimers. This 2A type includes not only the previous IIA subtype, but also some rare phenotypes characterized in specific families or group of families. Among them, phenotype IIC has been described so far in 12 patients belonging to 6 families of different origins. All diagnoses were asserted on the basis of abnormal vWF multimer profiles mainly characterized by a relative increase of the protomeric form associated with the lack of HMW multimers, suggesting the impairment of the multimerization process. The absence or decreased number of satellite bands in the

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**Fig 2.** Electrophoretic patterns of plasma vWF in SDS-2.5% agarose gel. Lanes 1 and 4, pool of normal plasmas; lane 2, patient A plasma; lane 3, patient B plasma. Numbers indicate the number of protomer repeats, with band 1 corresponding to the fastest migrating molecule (protomer). Arrows indicate satellite bands absent from the patients' plasma.

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**Fig 3.** Part of the nucleotide sequence gels of PCR-amplified exon 15. (A) Patient B and control sequences; boxed nucleotides represent the 3-bp insertion and the asterisk identifies the corresponding codon alteration. (B) The control and patient A sequences; the asterisks identify the single point mutation and the resulting codon change.
triplet unit, observed under high resolution electrophoretic conditions, has been also described as a specific feature of this subtype and may result from a decreased sensitivity to proteolysis. As like many other subtypes of vWD, subtype IIC has been found to be phenotypically heterogeneous, but the disease transmission observed within all affected families but one suggested the recessive inheritance of this particular category of type 2A vWD. Recessive type IIC vWD was assumed to occur in some families as the result of both a quantitative defect on one allele and a qualitative defect on the second allele. This was further supported by the allele segregation study performed in our laboratory on family B. We now report an additional patient with IIC phenotype, patient A, presenting a consanguinity history as an alternative cause of the expression of the disease. The previously suggested recessive inheritance of type IIC vWD is verified in both families A and B by the characterization at the DNA level of 3 candidate mutations in the two unrelated patients: one of a consanguineous origin was found homozygous for a single defect, whereas the second was shown to be a composite heterozygote.

In family A, the patient is homozygous for the missense mutation Cys623Trp and both her parents were probably heterozygous for this substitution inherited from their common ancestor. In family B, the patient has received from her mother the likely qualitative defect of vWF that is related to the insertion of Gly at position 625. Indeed, this vWF sequence alteration cosegregates with the double-peak cross immunoelectrophoretic (CIE) pattern observed in several family members (II2, II4, III3, and III4), including the propositus’ mother, with each peak corresponding to a population of vWF molecules (normal or mutant). Patient B has also inherited from her father another defect corresponding to an early translational stop at codon 711. Both are heterozygous for the 711ter mutation, as was the paternal aunt studied. Several nonsense mutations have been reported recently in severe vWD families: Arg365ter in exon 9, Val842ter in exon 19 resulting from a single cytosine deletion in exon 18, Arg1659ter in exon 28, Arg1852ter in exon 32, and Arg2535ter in exon 45. Therefore, it is likely that the not yet reported nonsense mutation we identified in the paternal side of patient B’s family is also responsible for the recessive quantitative defect of plasma vWF. Because the heterozygous state of such gene defects is not consistently reflected phenotypically by low vWF levels, it is not surprising that patient B’s father had a normal vWF level, whereas his step sister and his mother apparently had lower vWF levels. Thus, patient B appears to be a composite heterozygote for two recessive vWF defects: one quantitative (Tyr711ter), inherited from her father, likely belongs to type III vWF mutations, whereas the other (625insGly), qualitative, was inherited from her mother.
The vWF gene defects reported here are most likely at the origin of the disease in the two families studied because of their cosegregation with the phenotypic expression of type IIC vWD and of their localization within the vWF propeptide known to play an important role in multimer assembly. The causal relationship between the two qualitative defects found and the disease expression is also reinforced by the failure to detect similar alterations in 96 normal alleles. Furthermore, the two qualitative alterations potentially responsible for the IIC phenotype of vWD described here in two unrelated families are both localized in exon 15 and are only 2 aa apart. To our knowledge, no gene defect has already been identified in the other well-characterized type IIC vWD patients. In the type IIC Miami patient, the sequencing of exon 15 did not show any mutation on either allele. However, this Miami subtype is also characterized by an autosomal dominant inheritance, high levels of vWF:Ag, and the presence of HMW multimers in platelet vWF, features that more likely define another category in type 2A vWD, as suggested by the investigators.

The localization of both identified defects in the domain D2 of vWF propeptide underlines the critical role of this particular domain in the multimerization process. The secondary structure of vWF propeptide is still unknown and the Cysteine residues involved in disulfide bonds have not been yet identified. In the absence of such information one can only speculate on the vWF molecule alterations resulting from the two mutations described. The expression of recombinant vWF harboring these defects, in addition to verifying that the sequence changes described are responsible for type IIC vWD, should provide new insights into the understanding of the mechanisms involved in the multimerization process.

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