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## The molecular genetics of von Willebrand disease

A B S T R A C T

von Willebrand disease (VWD) is the most common inherited bleeding disease in humans, resulting in a clinical condition in which excessive mucocutaneous bleeding is the major manifestation. Significant progress has been made in the past decade in defining the molecular genetic basis of this condition. The gene that encodes von Willebrand factor (VWF) is located on chromosome 12p and comprises 52 exons spanning ~178 kb of genomic sequence. Of the two quantitative forms of the disease, types 1 and 3 VWD, significantly more is known of the molecular causation of severe type 3 disease in which a null VWF phenotype exists. Reports of gene deletions, insertions, nonsense mutations splicing defects and some missense mutations have all been documented in type 3 patients. In contrast, knowledge of the molecular pathology of type 1 disease is less advanced, although two recent, large population studies have indicated that a heterogeneous collection of missense mutations is associated with the disease in ~75% of cases. However, in some type 1 cases no mutations have been found in the VWF gene indicating that other genetic loci also contribute to this condition. In types 2A, 2B and 2M VWD, dominantly acting missense mutations predominate. In type 2A disease, two principal pathogenetic mechanisms have been defined: a failure to synthesize high molecular weight VWF multimers due to mutations in the VWF propeptide, D3 and A2 domains and the C-terminal knot region or an increased susceptibility to ADAMTS13-mediated proteolysis due to mutations adjacent to the cleavage site in the A2 domain. Types 2B and 2M disease are VWD variants in which gain-of-function and loss-of-function platelet-dependent phenotypes are documented, respectively. Both subtypes are due to heterozygous missense mutations affecting the glycoprotein Ib binding A1 domain of VWF. Finally, type 2N disease is a recessive disorder in which missense mutations affect the factor VIII binding domain at the N-terminus of the VWF monomer (D' and D3 domains).

Key words: von Willebrand disease: molecular genetics

**T**he adhesive glycoprotein von Willebrand Factor (VWF) plays two critical roles in normal hemostasis, mediating platelet adhesion to the exposed subendothelial matrix and protecting factor VIII from premature proteolysis by activated protein C.<sup>1</sup> Quantitative and/or qualitative abnormalities of VWF are responsible for the mucocutaneous inherited bleeding disorder, von Willebrand disease (VWD).<sup>2</sup>

### **Epidemiology and clinical features**

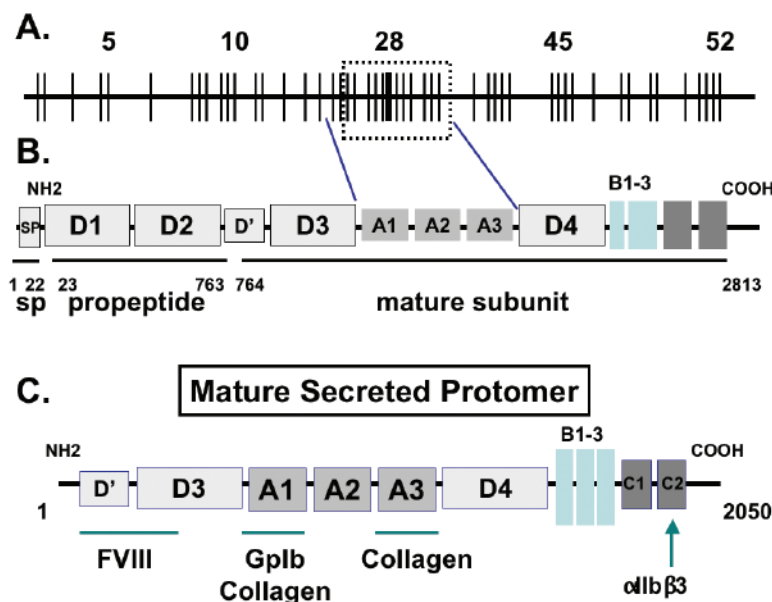
Von Willebrand disease is the most common inherited bleeding disease in humans, with a prevalence somewhere between 1% (findings from prospective epidemiological studies) and 1 in 10,000 (referrals to specialist medical centers).<sup>2,3</sup> In most instances, the clinical presentation of VWD involves an excessive mucocutaneous bleeding phenotype with easy bruising, epistaxes, bleeding

from wounds and prolonged post-procedural bleeding. In women with VWD, menorrhagia may be the only clinical manifestation. Soft tissue bleeding and hemarthroses are only encountered with severe VWF deficiency (plasma levels <0.01 U/mL).

The diagnosis of VWD requires consideration of three components: a personal history of excessive mucocutaneous bleeding, a set of laboratory abnormalities consistent with the diagnosis of VWD and a family history of excessive bleeding.

### **The von Willebrand factor gene**

The gene that encodes VWF is located on the short arm of chromosome 12 and the 52 exons of the gene are contained in a genomic construct that encompasses ~178 kb of DNA (Figure 1).<sup>4</sup> The exons range in size between 40 and 1379 base pairs (bp) in length, while the introns vary from 97 bps to



**Figure 1.** A. The VWF gene with 52 exons spanning ~178 kb of genomic DNA. Exons 23-34 of the gene are duplicated with 3% sequence variance on chromosome 22 in the form of a partial, non-processed pseudogene. B. The primary VWF translation product comprising a 22 amino acid signal peptide, a 741 residue pro-peptide and a 2050 residue mature subunit. C. The secreted VWF monomer comprising 2050 amino acids arranged in four repeat domains A-D. The secreted monomer has binding sites for the following ligands: factor VIII (D' and D3 domains); collagen (A3 and A1 domains); platelet glycoprotein Ib (A1 domain) and the  $\alpha IIb\beta 3$  integrin (C2 domain).

19.9 kb. The 22 amino acid signal peptide and 741 residue propeptide of von Willebrand factor are encoded by the first 17 exons of the gene while the mature secreted monomer of VWF and the 3' untranslated region are encoded by exons 18-52 in the 3' 100 kb of the gene. There are a number of repetitive sequences within the gene including 14 Alu repeats and an approximately 670 bp TCTA simple repeat in intron 40 that is highly polymorphic.<sup>5,6</sup>

The first VWF exon (250 bps) is a non-coding exon. There is a TAATTA, TATA box sequence at nt -32 in the promoter and a CCAAT element downstream of the CCAAT sequence. The transcriptional control of VWF expression is complex and appears to involve an interplay between a number of both positive and negative regulators. Binding of ETS, GATA and YY1 has been implicated in transcriptional upregulation of the locus whereas binding of NF1, Oct1, YY1 (to a non-CCAAT sequence) and E4BP4 act to repress VWF expression.<sup>7-10</sup>

In the upstream regulatory region of the gene two types of polymorphic elements have been characterized. At ~nt -650 there is a polymorphic dinucleotide repeat sequence and at nts -1234, -1185 and -1051 there are three single nucleotide polymorphisms (SNPs) that usually co-segregate in two haplotypic groups. Study in normal subjects with blood group "O" has shown an association between these regulatory sequence haplotypes and the plasma levels of VWF.<sup>11,12</sup>

#### **Additional considerations in genetic studies of von Willebrand disease**

To complicate genetic investigation, exons 23-34 of the chromosome 12 sequence are duplicated in the form of an unprocessed partial pseudogene on chromosome

22 that shows ~3% sequence divergence from the chromosome 12 locus.<sup>13</sup> Amplification of this region of the chromosome 12 VWF gene needs to take into account both the normal vs pseudogene sequences and the frequent polymorphisms present in the VWF gene.

The other challenge in interpreting genetic information from the VWF gene is the level of polymorphic variation of the gene sequence. Currently, 81 SNPs have been located within the coding region of the VWF gene, 31 of which alter the encoded amino acid. In some instances, it still remains unclear whether these changes are phenotypically neutral or whether they are contributing either directly or indirectly to a qualitative or quantitative defect of VWF. This is an area of ongoing investigation and the most up to date information is available at the International Society on Thrombosis and Haemostasis' VWF website available at URL <http://www.shef.ac.uk/vwf/>.

#### **Type 1 von Willebrand disease**

Type 1 VWD, a mild to moderate quantitative deficiency of normal VWF, accounts for ~80% of all VWD cases. While this is the most common variant of this condition, it is also the most problematic to diagnose, especially in its milder form. The recognized influence of factors such as ABO blood type<sup>14</sup> and "stress",<sup>15</sup> on the plasma levels of VWF, significantly complicate the diagnosis of type 1 VWD except when the plasma level is <0.20 U/mL. This complexity, along with the relatively mild bleeding manifestations associated with minor VWF reductions has resulted in a recent call for a re-evaluation of the diagnostic criteria for type 1 disease.<sup>16,17</sup> This controversy remains an ongoing issue with the VWF Scientific and Standardization Subcommittee of the Inter-

national Society on Thrombosis and Hemostasis.

In the diagnostic hemostasis laboratory, type 1 VWD manifests as reductions of all three components of the FVIII/VWF complex: VWF:Ag, VWF:RCO and FVIII:C, although the FVIII:C level is usually higher than the VWF plasma levels. While the definition of type 1 disease requires that the VWF is qualitatively normal, there is recent evidence that some type 1 cases have subtle losses of the highest molecular weight VWF multimers, likely as a result, at least in part, of increased ADAMTS13-mediated proteolysis. However, these abnormalities are unlikely to be appreciated in most diagnostic laboratories using their routine multimer analysis.

Type 1 VWD is an autosomal dominant trait that manifests incomplete penetrance and variable expressivity.<sup>18</sup> For these reasons, the identification of a family history of the condition may be problematic in many cases.

Until very recently, the underlying molecular genetic pathology of type 1 VWD was very poorly characterized. There are some uncommon instances in which relatively severe type 1 VWD (plasma VWF level <0.20 U/mL) is the result of highly penetrant dominant mutations that disrupt VWF biosynthesis.<sup>19,20</sup> However, over the past three years, two large type 1 VWD studies in Europe and Canada have completed an initial assessment of the genetic defects that result in a large spectrum of type 1 VWD cases. Overall, these studies have documented two major findings: that a substantial proportion of type 1 VWD cases are not linked to the VWF gene locus and that in those cases that do result from changes within the VWF gene, 75–80% of cases are due to a variety of different missense mutations. Additional types of mutations found in these patients have included splicing errors, nonsense mutations and small insertions and deletions. These mutations have been distributed throughout the VWF gene from codon 19 in the signal peptide to codon 2722 in the C-terminal CK domain. In addition, some sequence variations have been localized to the 5' non-coding region and may adversely affect transcription.

Prominent among the missense mutations associated with type 1 VWD is the Tyr584Cys substitution that has been found in 8–20% of type 1 cases in Europe and North America.<sup>21,22</sup> The penetrance of this mutation is ~75% and there are reports that the mechanisms underlying the low VWF levels associated with this mutation involve both intracellular protein retention and enhanced ADAMTS13-mediated VWF proteolysis.<sup>21,23</sup>

Other commonly occurring missense changes found in the European and Canadian type 1 VWD populations include Arg845Gln, Arg924Gln, Arg1205His, Arg1315Cys and Arg1374Cys. These observations illustrate the genetic heterogeneity that underlies this condition and also emphasize the *blending* of type 1 VWD alleles with alleles for other VWD variants including the common

type 2N Arg854Q allele<sup>24</sup> and the Arg1205His VWD Vicenza mutation.<sup>25,26</sup> While the prospect of gene-based diagnosis of type 1 VWD is still not feasible, the development of enhanced genetic testing modalities and a better understanding of the pathogenic significance of the sequence variability in the VWF gene may eventually enable the evaluation of a panel of prevalent type 1 mutations at some point in the future.

### **Type 3 von Willebrand disease**

In contrast to type 1 disease, type 3 VWD represents the manifestation of severe VWF deficiency, with frequent mucocutaneous and musculoskeletal bleeding problems. Type 3 disease has a prevalence of ~1 in 500,000.<sup>2</sup> In the diagnostic laboratory, type 3 disease, shows VWF:Ag and VWF:RCO levels that are either undetectable or <0.02 U/mL. Factor VIII levels are always <0.10 U/mL.

While the classical description of type 3 VWD suggests that this is an autosomal recessive trait with heterozygous parents manifesting no clinical or laboratory signs of the condition, there is now a growing appreciation that in some cases, one or even both parents may manifest consequences of a null type 1 VWF allele. In type 3 patients, the genetic pathology represents either homozygosity or compound heterozygosity for two VWF null alleles.

The initial molecular genetic characterization of type 3 VWD was described in patients with gross deletions of the VWF locus, some of whom had also developed alloantibodies against the infused therapeutic VWF protein.<sup>27,28</sup> However, subsequent studies of type 3 VWD cohorts have identified a variety of mutation types, all of which, in combination, result in a null phenotype. Thus, the original *Aland Island* VWF mutation in the families that were described by Erik von Willebrand in 1926 is a single cytosine deletion in exon 18, present in the homozygous form in type 3 VWD patients.<sup>29</sup> The exon 18 cytosine deletion has also been found in type 3 patients in several other ethnic groups as have some nonsense mutations occurring at hypermutable CpG sequences (ie. Arg1659Stop and Arg2535Stop) (Figure 2). Interestingly, in addition to the collection of nonsense, insertion and deletion mutations that one might expect to be associated with null alleles, there are also a number of missense mutations that result in this phenotype. These often involve cysteine residues and appear to be clustered in either the pro-peptide or C-terminus of the protein suggesting that they severely interfere with either dimer or multimer assembly and abolish VWF secretion.<sup>30</sup>

### **Type 2 von Willebrand disease:**

The remaining ~20% of VWD cases represent the four qualitative type 2 variants: 2A, 2B, 2M and 2N.

### Recurrent Type 3 von Willebrand Disease Mutations

**R2535X**

Holland, Turkey, Sweden, UK, Italy, Germany

**R1659X**

Spain, Japan, Iran

**Exon 18 2435delC**

Sweden, Italy, Germany, Poland

**Figure 2.** Details of three of the common recurring type 3 VWD mutations and the geographical locations where they have been documented.

#### Type 2A VWD

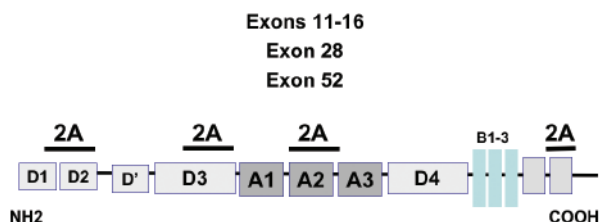
Type 2A VWD is the second most prevalent form of VWD presenting with signs and symptoms that are indistinguishable from type 1 disease. In the laboratory, both type 2A and 2M VWD show marked disparities between the functional VWF:RCO (or VWF:CB) and immunologic VWF:Ag results, with ratios of <0.5–0.7 being strongly suggestive of a defect in the platelet-dependent function of VWF. In addition to the distorted VWF:RCO to VWF:Ag ratio, type 2A VWD plasma also shows a loss of high molecular weight VWF multimers. In this variant form of VWD, two pathogenetic mechanisms have been defined: either defects in the biosynthetic processing of VWF multimers (secondary to mutations affecting the C- and N-terminal domains of VWF involved in dimer and multimer formation, respectively) or the synthesis of VWF that is more susceptible to ADAMTS13-mediated proteolysis (due to mutations affecting the VWF A2 domain).<sup>31,32</sup> The structural basis for these pathogenetic mechanisms can likely be explained by the location and relative disruptive nature of the mutation.<sup>33</sup>

To date, >50 different mutations have been characterized in type 2A VWD. In most instances, these are dominantly inherited missense changes involving one of four regions of the VWF protein (Figure 3): the VWF propolypeptide (corresponding to a type 2A, subtype IIC variant multimer pattern); the D3 domain (type 2A, subtype IIE); the A2 domain ("classical" type 2A VWD) and the C-terminal knot region of the protein (type 2A, subtype IID). Some type 2A mutations have been documented multiple times (ie. Arg1597Trp and Ser1506 Leu).

### Type 2A von Willebrand Disease

Loss of Platelet-dependent Function  
(abnormal multimers)

~50 mutations



**Figure 3** Type 2A VWD mutations localize to four regions of the VWF protein: the pro-polypeptide (D1/D2), the N-terminal region of the VWF monomer (D3), the A2 domain and the C-terminal knot region.

#### Type 2B and 2M VWD

Types 2B and 2M disease represent gain- and loss-of-platelet dependent function variants, respectively. Type 2B VWD shows a loss of high molecular weight VWF plasma multimers and, most characteristically, an enhanced sensitivity to ristocetin-induced platelet agglutination of platelet rich plasma. In contrast, the multimer pattern in type 2M disease is normal, and there is reduced responsiveness to ristocetin. The mutations resulting in these two distinct phenotypes are clustered in exon 28 of the gene and alter residues in the GPIb binding, A1 domain of VWF (Figure 4). Placement of the 2B and 2M VWD mutations on a structural model of the A1 domain indicates that they are located in distinct regions of the domain that presumably either enhance (type 2B) or abrogate (type 2M) the potential for interacting with the GPIb platelet receptor.<sup>34</sup>

To date, 22 different missense mutations have resulted in type 2B VWD and one 2B mutation represents the insertion of a methionine residue. All of these changes are clustered in the A1 domain of the protein. Sixteen different type 2M mutations have been characterized in the same domain of the protein.

The fact that all these mutations are localized within a single 1.3 kb exon of the VWF gene (exon 28) indicates that it is relatively easy to perform confirmatory molecular genetic testing for these phenotypes with direct sequencing of a single PCR product. This strategy can also be used as an alternative to platelet and plasma mixing studies to differentiate between type 2B VWD and platelet-type or pseudo-VWD in which the gain-of-function genetic defect is located in the gene encoding the platelet GPIIb/IIIa receptor.<sup>35</sup>

### Type 2B and 2M von Willebrand Disease

**Type 2B:** Gain of platelet-dependent function

**Type 2M:** Loss of platelet-dependent function

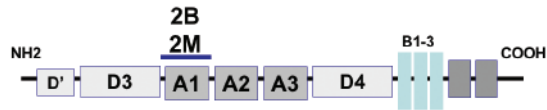
#### Type 2B VWD

23 mutations

22 missense : 1 insertion of Met

#### Type 2M VWD

15 missense mutations



**Figure 4.** Type 2B and 2M mutations are located in exon 28 in the glycoprotein Ib binding A1 domain of the VWF monomer.

#### Type 2N VWD

Type 2N VWD represents an autosomal recessive trait in which mutations in the D' and D3 domains of the protein disrupt the factor VIII binding potential of VWF (Figure 5). In type 2N disease, the only laboratory manifestation may be a low FVIII:C, thus placing this condition as a differential diagnosis of mild hemophilia A or the carrier state for hemophilia A.<sup>24</sup> The phenotypic manifestation of type 2N disease requires the inheritance of two mutant VWF alleles, with all patients being either homozygous or compound heterozygous for two type 2N missense mutations, or occasionally being compound heterozygous for a type 2N allele and a VWF null allele.

The most common type 2N allele is the Arg854Gln mutation that is associated with FVIII:C levels of ~0.20 U/mL and a desmopressin response that is therapeutically useful. In contrast, other recurring 2N mutations such as Arg816Trp and Thr791Met result in lower FVIII:C levels (~0.10 U/mL) and a less satisfactory response to desmopressin infusion.

All of the type 2N VWD mutations have been missense mutations located between exons 18–25 of the VWF gene. Thus, while a factor VIII binding assay still represents the definitive phenotypic test for this variant, sequencing of these eight small VWF exons (104–161 bps) can also be performed to confirm this diagnosis.

#### The future of VWD molecular genetics

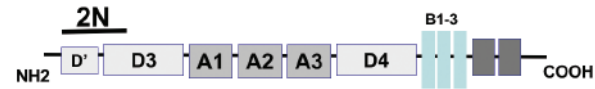
Molecular genetic studies of a condition such as VWD can be pursued with one of several basic or applied objectives in mind. Basic science outcomes can include a better understanding of structure/function correlates, or an appreciation of types and patterns of disease causing mutations. Studies during the past two decades have fulfilled these objectives for all forms of VWD, although

### Type 2N von Willebrand Disease

#### Reduced FVIII Binding

19 missense mutations

Exons 18-25



**Figure 5.** Type 2N missense mutations are all located in the N-terminal factor VIII binding region of the factor VIII monomer (D' and D3 domains), encoded by VWF exons 18–25.

### Genetic Diagnosis of von Willebrand Disease

<b>Type 1</b>	-	<b>Possible screening in future</b>
<b>Type 3</b>	-	<b>For prenatal diagnosis</b>
<b>Type 2A</b>	-	<b>Unnecessary</b>
<b>Type 2B</b>	-	<b>Confirmatory (exon 28)</b>
<b>Type 2M</b>	-	<b>Confirmatory (exon 28)</b>
<b>Type 2N</b>	-	<b>Often helpful (exons 18-25)</b>

**Figure 6.** Potential applications of molecular genetic testing strategies for the diagnosis of VWD.

much still needs to be learnt about the definitive pathogenetic mechanisms responsible for type 1 disease.

In contrast, the utility of molecular diagnostics for VWD remains limited. While the diagnosis of VWD still rests critically upon the clinical picture and the results of tests performed in the clinical hemostasis laboratory, there is a growing potential for the incorporation of molecular genetic studies to confirm or refute several types of the disease (Figure 6). This potential is most obvious for types 2B, 2M and 2N disease, but the recurrent nature of a significant number of type 1 mutations suggests that it may be possible, in the future, to design a genetic screening approach as an adjunct to the routine phenotypic testing for this frustrating variant. With the significant temporal variability of VWF levels, the

stability of the VWF genotype offers a complementary diagnostic strategy that is becoming increasingly feasible with advances in genetic testing methodologies.

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