

# Molecular Diagnosis of von Willebrand Disease

Changgeng Ruan

*Jiangsu Institute of Hematology, Suzhou University Medical College, China*

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## Abstract

von Willebrand disease (vWD) is one of the most common inherited human bleeding disorders, which is caused by quantitative or qualitative defects of von Willebrand factor (vWF). vWF is a highly multimerized glycoprotein that promotes platelet adhesion and aggregation at a high shear rate, while also acting as a carrier of coagulation factor VIII. vWD has been subdivided into three categories, which reflect their pathophysiology. Type 1 and type 3 vWD reflect partial or complete deficiency of vWF, whereas type 2 vWD reflects qualitative defect of vWF. The ability of vWF to interact with its platelet receptor and factor VIII, and the analysis of the multimeric composition of vWF are essential to identify patients with different vWD subtypes. The prevalence of different vWD subtypes was reported in the literature. In the past years, ninety-one patients with vWD were consulted in our institution. Of all the vWD patients, 56 (61.5%) belong to type 1, 26 (28.6%) type 2 and 9 (9.89%) type 3. The analysis of vWF gene was performed in some type 2 and type 3 vWD by denature gradient gel electrophoresis and sequencing. We have found six cases of point mutations of vWF gene, Ala737→Glu, Gly 22→Glu, Met37 Val and Ser71→stop codon. Substitutions, are first reported in international database. We constructed an expression plasmid pSVA737EvWF containing full length of cDNA of vWF which included the Ala737 Glu substitution by site-direct mutagenesis. The structure of recombinant vWF within transfected COS-7 cells and the secretion of high-molecular-weight (HMW) multimers were similar to wild-type vWF. HMW forms of vWF multimers were absent in plasma but present in platelets. The mutation corresponds to the group II type 2A vWD characterized by normal secretion of all vWF multimers.

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von Willebrand disease (vWD), which is caused by the qualitative and quantitative defects in von Willebrand factor (vWF), is one of the most common inherited human bleeding disorders. vWF plays an important role in primary hemostasis by mediating the adhesion of platelets to sites of vascular injury through its binding to specific platelet membrane glycoproteins and to constituents of exposed connective tissue. In addition, vWF is also a carrier protein for blood clotting factor VIII (FVIII), and this interaction is required for normal FVIII survival in the circulation.

The human vWF gene, which has been localized to chromosome 12 (12p12-pter) [1,2], spans approximately 180 kb and is composed of 52 exons [3]. The 8.9 kb mRNA encodes a primary translation product which is composed of a 22 amino acid signal peptide, a 741 residue pro-peptide and a 2050 amino acid mature vWF subunit. vWF is synthesized in both megakaryocytes and endothelial cells by a complex multistep process that results in the assembly of multimers of up to 100

subunits. These high molecular weight (HMW) multimers appear to be most effective in platelet binding.

The increase in information about the molecular structure of vWF has led to a simplified classification of vWD. In 1994, according to the clinical phenotype, pathological characteristics, and molecular pathogenesis of vWD, the International Thrombosis and Hemostasis Committee proposed a new classification standard that divides vWD into 3 categories [4]. Type 1 refers to a partial quantitative deficiency of vWF and autosomal dominant inheritance. Type 1 is characterized by a concordant reduction in vWF antigen, ristocetin cofactor activity, and FVIII activity, but the structure of the vWF multimers is normal. Type 2 refers to qualitative deficiencies of vWF and is generally inherited in a dominant manner although rare cases of apparently recessive inheritance have been reported. Type 2 contains subtype 2A, 2B, 2N and 2M, and is characterized by a defect in vWF function and often also quantity. Type 2A and 2B are frequently associated with a disproport-

tionately low level of ristocetin cofactor activity relative to vWF antigen, and a decrease or absence of the largest vWF multimers. Type 2M and 2N are associated with specific defects in platelet and FVIII binding functions, respectively. Type 3 is usually defined as autosomal recessive; this is not a completely consistent finding.

The prevalence of von Willebrand disease ranges from 3 or 4 per 100,000 to as high as 1.3% of the population [5-7]. Among the different vWD types, type 1 is the most frequent (60-80%); all type 2 vWD variants together are 15 to 40%, whereas type 3 is diagnosed in 5 to 10% of vWD patients. The prevalence of vWD in China is unknown. In the past years, patients with various types of inherited bleeding disorders, such as hemophilia A (HA), hemophilia B (HB) Glanzman's thrombasthenia (GT) and vWD were assessed in our hospital. Ninety-one (42.13%) of them were vWD patients. Of all the vWD patients, 56 (61.5%) were type 1, 26 (28.6%) type 2 and 9 (9.89%) type 3. All vWD is caused by the mutation at the vWF locus. Given the complexity of vWF biosynthesis, secretion, and function, defects at a number of genetic loci could potentially result in the different variants and subtypes of vWD. Analysis of the vWF gene was performed in a subset of patients with type 2 and type 3 vWD by denaturing gradient gel electrophoresis (DGGE) and sequencing, and we found six cases of point mutation in these patients with vWD (Table 1).

### Type 1

Type 1 vWD is the most frequent and the mildest form of vWD. The diagnosis of type 1 vWD requires three factors: a significant mucocutaneous bleeding history, a positive family history with dominant inheritance and low levels of vWF (antigen and activities) with normal multimeric structure. However, these criteria are not met in many patients with type 1 vWD who may not present a significant bleeding symptoms and therefore never receive the treatment to raise their low vWF levels. In 1996, the Subcommittee of the SSC on vWF of the International Society of Thrombosis and Haemostasis proposed the use of the term *possible vWD type 1* for patients whose laboratory tests are compatible

with type 1 vWD and who have either a bleeding history or a positive family history of type 1 vWD. This uncertain definition, however, did not solve the problem of the diagnosis of type 1 vWD in many laboratories. A study entitled Molecular and Clinical Markers for Diagnosis and Management of type 1 vWD is currently under the investigation in Europe. In addition to the bleeding history and basic laboratory tests for vWF parameters, the advanced phenotypic tests (vWF: collagen binding activities, vWF: FVIII binding activities, and platelet vWF) and the gene mutation analysis will be performed in this study.

### Type 2A

Type 2A vWD is the most common qualitative variant, accounting for approximately 10-15% of all vWD diagnosis. Most of the mutations responsible for type 2A vWD cluster within exon 28, which is 1.4kb in length, and is the largest of all exons. It encodes the entire A1 and A2 repeats, including the proteolysis site of vWF. At least 24 missense mutations are known to cause dominant type 2A vWD. Twenty of these mutations are within the vWF A2 domain and four missense mutations are in the vWF A1 domain. Analysis of the type 2A vWD phenotype and study of its molecular pathogenesis will contribute to further understanding of vWF structure and function and direct gene diagnosis.

Three mutations have been found in 14 patients with type 2A vWD patients that were diagnosed in our institute [8,9]. They are Arg611→His, Ala737→Glu and Arg834→Trp and are all hot point mutations locating on the GpC island. Arg611→His substitution has been reported in 2 unrelated families [10], and it locates on the A1 domain of vWF. Most type 2B vWD mutation have been found in this domain, but the mutation reported here is associated with decreased ristocetin cofactor activity and the absence of large and intermediate vWF multimers. Arg611→His mutation results in a decreased affinity of vWF for glycoprotein Ib. This type is often identified as a type 2A vWD variant. Arg834→Trp is the most common mutation in 2A vWD. In vitro expression confirmed that Arg834→Trp did not disturb the assembly and secretion of multimers of vWF in cells, but the absence of large and intermediate vWF multimers was due to the high susceptibility of vWF to proteolysis in plasma [11].

Two patients from the same hemorrhage family showed prolonged bleeding time, markedly decreased ristocetin induced platelet aggregation, decrease of vWF: Ag and FVIII:Ag, and absence of both large and intermediate molecular weight forms of von Willebrand factor multimers in plasma, and were studied recently. A novel missense mutation of C to A was detected, which resulted in Ala737→Glu substitution. The type 2A vWF family with Ala737→Glu mutation showed the characteristic of autosomal dominant inheritance. Among 19 members within 4 generations, 9 possessed similar bleeding symptoms. The mutation is located within the

**Table 1.**

Mutations of von Willebrand Factor Gene in Chinese Patients with vWD.

Type	Nucleotide substitution	Amino acid substitution
2A	G4121A	Arg611→His
2A	C4499A	Ala737→Glu
2A	C4789T	Arg834→Trp
2N	G2354A	Gly22→Glu
2N	A2398G	Met37→Val
3	C212A	Ser71→stop code

A2 domain of vWF, within which most type 2A vWD are clustered. The Ala737→Glu substitution is considered a new mutation in the vWD database [12].

We constructed an expressing plasmid pSVA737EvWF containing the full length cDNA of vWF which included the Ala737→Glu substitution by site-direct mutagenesis. Recombinant vWF containing the candidate mutation was transiently expressed in COS-7 cells. Compared with wild type, recombinant vWF expressed in conditioned media was 76.4%, while in cell lysate was 98.8%. The multimer pattern of extracellular pSVA737EvWF was indistinguishable from that of the wild type, and both were comprised of many molecular weight multimers in the platelets from patients indicated that the mutation didn't disturb the conformation of structure and secretion of vWF. The absence of large and intermediate multimers in plasma is possibly due to mutation in the vWF gene where a neutral amino acid is replaced by an acidic amino acid, resulting in a conformational change and an abnormally increased susceptibility to proteolysis in plasma. vWF mutations produce type 2A vWD by at least two mechanisms. One class of mutations (group 1) causes defective intracellular transport of vWF and impairs the assembly, storage, and secretion of large vWF multimers in both the plasma and the platelet compartments. Group 2 mutations do not interfere with vWF assembly or secretion but render the multimers more sensitive to proteolysis in plasma. The new mutation Ala737Glu corresponds to the group II type 2A vWD [13]. Combined with gene mutation in vWD patients, the use of site-directed mutagenesis and expression of vWF will help us understand the function of vWF and give us a model for studying the molecular pathology in other bleeding disorders.

### Type 2N

Type 2N vWD patients have a phenotype similar to the patients with mild hemophilia A. This vWD phenotype is characterized by normal levels of vWF:Ag and vWF:Rco, and normal multimeric structure but low plasma FVIII levels. Type 2N vWD is caused by decreased plasma half-life of FVIII, which can not bind to vWF as a consequence of an intrinsic abnormality of vWF. The FVIII binding domain has been localized to the N-terminal fragment composed of 272 amino acid residues, encoded by exons 18-23 of the vWF gene. Mutations detected in type 2N vWD were all located within exons 18-20 of vWF gene. We found two novel candidate missense mutations of this type. One abnormal pattern is in exon 18 of the vWF gene of type 2N pedigree. DNA sequencing demonstrated a heterozygous G to A transition, substituting glutamic acid to glycine in position 22 [14]. This novel missense mutation creates a new restriction site for the enzyme SacI. The family study showed that the mutation originated from the mother. For another candidate, we screened exon 18-20 of the vWF gene from 22 patients with mild

phenotype of hemophilia A by DGGE and found that one fragment of exon 18 showed an abnormal electrophoretic pattern. DNA sequencing demonstrated an A to G transition at nucleotide 2398 in exon 18, substituting Met to Val at position 37 in the mature vWF subunit.

### Type 3

Type 3 vWD represents a severe form of the disease with a nearly complete deficiency of vWF. Type 3 (severe) vWD is caused by impaired biosynthesis of vWF and is characterized by immeasurable levels of vWF in plasma and platelets. Because vWF is also the carrier of FVIII, plasma levels of FVIII are very low (1-10%). As a consequence, patients with type 3 vWD have a severe bleeding tendency, characterized not only by mucocutaneous hemorrhages but also by hemarthroses and hematomas like those observed in severe hemophilia. The inheritance pattern of type 3 vWD is autosomal recessive. The molecular defects associated with type 3 vWD are total or partial vWF deletions, nonsense, splicing, and frameshift mutations. A mutation was detected in a type 3 vWD patient by our lab [15] and was a nonsense mutation of C to A transition at nucleotide 212 in exon 3 of vWF gene, introducing a stop codon at 71. This mutation created a Xba I restriction enzyme site and eliminated a Taq I restriction site. The patient was homozygous for the mutation and his parents were heterozygous, whose FVIII: Ag, vWF: Ag and bleeding time were normal.

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