# The importance and complications of Sequencing of von Willebrand gene in von Willebrand disease

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**Abstract**

Genetic testing in patients with von Willebrand disease completes phenotypic testing with an aim to confirm the von Willebrand factor defect at a molecular level. Structure of the *VWF* gene was described 30 years ago; since then a large number of mutations leading to VWD have been described in this gene. Thanks to describing these mechanisms it is possible to understand the pathogenesis of the most common congenital bleeding disorder.

In the Slovak Republic genetic testing is still not a routine part of VWD diagnostics. The National Center of Hemostasis and Thrombosis in Martin is the first department in Slovakia which has begun genetic testing of patients with VWD. Sequencing of the *VWF* gene has many limitations which are referred in more details within this article. Therefore, we decided to use the methods of new generation sequencing in combination with Sanger sequencing. We believe that soon we will have the first results which will help us to identify the possible cause of VWD in these patients.

**Key words:** *von Willebrand disease, von Willebrand factor, genetic testing, new generation sequencing, mutation*

## Characterisation of VWD

Von Willebrand disease (VWD) is considered to be the most common inherited bleeding disorder with an estimated prevalence about 1% of population. The prevalence based on clinical data is much lower, about 0.05 % people (1). In Slovakia the prevalence of patients with VWD is 11.2 per 100 000 (2).

The cause of VWD is an inherited defect of concentration, structure, or function of von Willebrand factor (VWF). Total deficiency or functional defect may be due to reduced or defective synthesis or accelerated degradation of VWF in the bloodstream (3). VWF is large plasmatic glycoprotein. In human plasma it is present as a series of polymers known as VWF multimers consisting of a variable number of subunits. Each VWF multimer subunit has binding sites for receptor glycoprotein (GP) Ib on unactivated platelets and receptor GP IIb/IIIa on activated platelets. This facilitates adhesion and platelet aggregation; therefore, it is important for normal platelet function. In addition, VWF is a carrier for the procoagulant factor FVIII which protects FVIII against rapid clearance, thereby increasing its half-life in plasma. For this reason VWF is essential for primary (platelet-mediated) and secondary haemostasis (mediated by coagulation factors) (4). Malfunction in one of these processes may lead to VWD that is mainly associated with excessive mucocutaneous (bruising, epistaxis, menorrhagia, gingival bleeding) and postoperative bleeding. In the case of severe defects,

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where the plasma concentration of FVIII is reduced secondary, there may also occur a bleeding that is more typical for coagulopathies such as haemophilia (muscular haematomas, hemarthrosis) (5).

Current VWD classification proposed by the International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee on VWF (ISTH-SSC VWF) divides VWD into 6 types – 1, 2A, 2B, 2N, 2M, 3. Types 1 and 3 are quantitative VWF deficiencies (partial and complete) and type 2 with four subtypes represents qualitative VWF deficiency (6).

## Genetic background

The gene encoding VWF was cloned and described by four independent groups in 1985. It is located on a short arm of the chromosome 12 at the position 12p13.31 with a total length of 178 kb. Its 52 exons encode 8.8 kb mRNA and a protein composed of 2813 amino acids. Normal gene product consists of precisely arranged repeating domains. Domains are stored in order from amino terminus S-D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK which are responsible for VWF functions (Fig.1) (6). The propeptide, i.e. the D1-D2 domain, has a role in the multimerization process. In the Golgi apparatus the dimers are formed by disulfide bridges at the N-terminus of the subunits, i.e. in the D´-D3 region. In addition, the D´ -D3 domains contain a binding site for the coagulation factor FVIII. The A1 domain is the only known binding site for the GP Ibα platelet receptor and provides a binding site for collagen type VI. The ADAMTS13 metalloprotease cleavage site is located in the A2 domain. Type I and III fibrillar collagens have a binding site in the A3 domain. Multimeric VWF requires a complex post-translational biosynthesis. After translocation into the endoplasmic reticulum the signal peptide is cleaved off and the pro-VWF dimerizes via disulfide bridges between the cysteine residues of the C-terminal CK domain (7).

In humans on the long arm of chromosome 22 at position 22q11.22-q11.23 there is a partial VWF pseudogene. Pseudogene sequence shows 97 % similarity to *VWF* exons 23–34

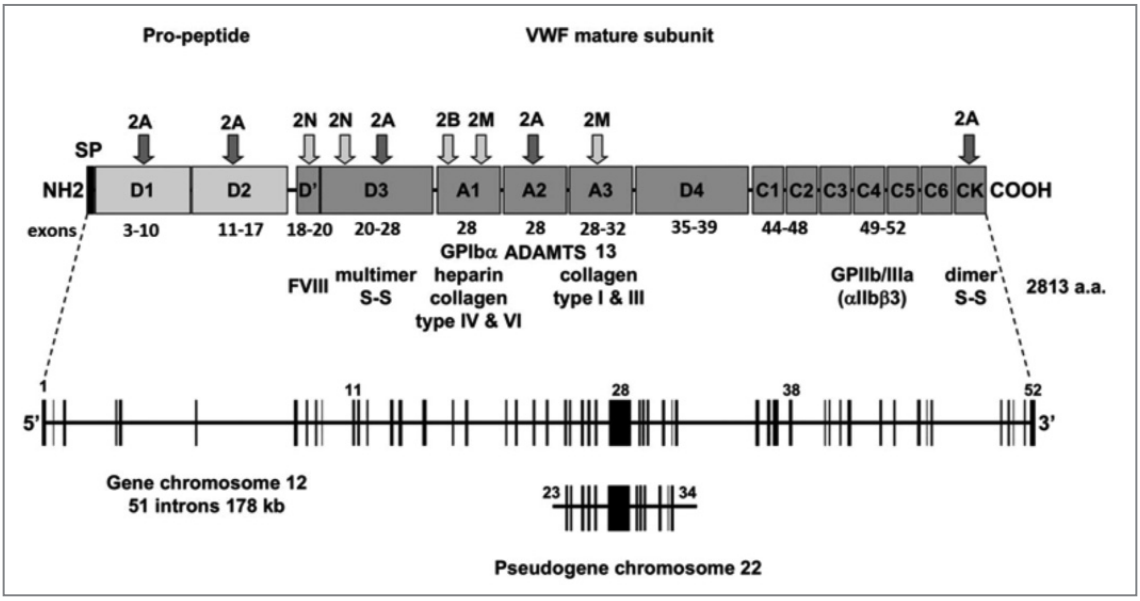


**Fig. 1.** Simplified structure of VWF precursor with labeled binding sites for the most important ligands. The arrows show the most frequent positions of mutations causing type 2 VWD. Mutations associated with types 1 a 3 VWD are dispersed throughout the VWF gene. Abbreviations: GP-glycoprotein, a.a – aminoacid, FVIII – coagulation factor FVIII (7).

sequences. The pseudogene contributes to VWD gene mutation spectrum by gene conversion and, in addition, in the corresponding region of exon 28, 12 polymorphisms were also found (8).

## Spectrum of mutations

During more than 30 years, since the *VWF* gene sequence was described, hundreds of mutations in the *VWF* gene have been discovered and described. In the ISTH-SSC VWF Online Database 589 mutations in the *VWF* gene have been registered so far (Tab.1). Most identified mutations are heterozygous missense mutations, small deletions/insertions, splicing and nonsense mutations in the coding region, promoter and splicing sites. Mechanisms inducing VWD due to these mutations have been demonstrated in *in vitro* or *in vivo* studies. However, many of the identified mutations remain candidate mutations because their influence on VWD induction has not been elucidated yet (9, 10).

**Table 1** Number of mutations in *VWF* gene registered in International database of the International Society on Thrombosis and Haemostasis (available online: www.ragtimedesign.com/vwf/mutation/)

| type of VWD | number of *VWF* mutations |
|---|---|
| **1** | 153 |
| **2A** | 104 |
| **2B** | 56 |
| **2M** | 31 |
| **2N** | 60 |
| **3** | 131 |
| **unclassified** | 32 |

*Type 1 VWD (AD inheritance)*

Type 1 represents the most common form of VWD (40-70% of all cases). It is defined as a partial quantitative VWF defect with mild to moderate bleeding symptoms. The VWF (VWF: Ag) plasma level varies between 5–50%, the functional activity of VWF and often also the FVIII is reduced proportionally (6).

Mutations in the *VWF* gene were identified in 60–70% of patients with type 1 VWD. They are located throughout the gene. Approximately 50% of the pathogenic variants in this type VWD are located between exons 18 and 28 (11). Various mutation mechanisms characteristic for VWD type 1 can cause decreased VWF production, reduced secretion, or increased clearance of VWF. Decreased VWF production was mostly described in patients with a null allel where the null allele does not have a gene product or the product does not have a proper functional activity. Null allele heterozygosity results in protein production from only the non-mutated allele, resulting in only 50% of normal expected production. Defects in secretion include preservation of VWF in the endoplasmic reticulum or Golgi apparatus, reduced exocystosis from Weibel-Palade bodies, or degradation of mutant VWF by the proteasome, although VWF production may be normal. Sequence variants of unknown significance are often identified and no *VWF* mutation was found in 35% of VWD type 1 patients. Therefore, recent studies have confirmed previous considerations that type 1 represents a complex genetic trait exhibiting both allelic and locus heterogeneity (6).

*Type 2 VWD (2A/IIA, 2A/IID, 2A/IIE, 2B, 2M – AD interitance, 2A/IIC, 2N – AR inheritance)*

Type 2 VWD with its 4 subtypes represents approximately 25% of all VWD cases. The relative frequency of the subtypes in the European population is 2A> 2N> 2M> 2B. The subtypes are characterized by various VWF malfunctions. VWF plasma level may be normal or reduced. Also FVIII plasma level may be normal or decreased. Type 2A is associated with defect in plasma VWF multimer as a result of defective intracellular multimerization or increased VWF susceptibility to ADAMTS13 protease cleavage. A tertiary category for 2A VWD includes mutations in the most common locations: the A2 domain (IIA), D3 domain (IIE), D2domain (IIC), and CK domains (IID). Mutation analysis can help in understanding of disease process in 2A VWD either through an analysis of exon 28 alone or, ideally, of the entire *VWF* gene (6, 10). In type 2B the gain-of-function mutations in the A1 domain cause VWF more active and can bind GP Ib even without its activation by high shear forces or ristocetin. In addition to the A1 domain, the mutations associated with VWD type 2B were also located in the D3 domain and at the C-terminal end of the chain. Mutations associated with the type 2M VWD are localized throughout the A1 domain and exhibit a defect in VWF binding to GP Ib or collagen. Mutations located in domains D2, D´and D3 affect the binding of VWF to FVIII. Since the FVIII: VWF plasma concentrations are maintained at a nearly constant ratio of 1:50, 1 FVIII molecule per 50 VWF monomers, the 2N phenotype only occurs in the case of homozygous mutations for FVIII defective binding or in the case of compound heterozygosity for defective FVIII binding and VWF and other *VWF* mutation (6,10).

*Type 3 VWD (AR inheritance)*

Patients with type 3 have undetectable plasma VWF levels and have a severe bleeding phenotype. Most patients do not produce VWF at all as a result of homozygous or compound heterozygous null alleles. Some patients are homozygous or heterozygous for missense mutations. These patients normally produce VWF but mutant VWF is not released from endothelial cells or is subjected to a very rapid clearance (6).

## Importance of genetic testing in VWD

If all VWF assays are used, the genetic testing for screening mutations only confirms the patient's phenotype. On the other hand, if some of these phenotypic assays are not available, then the identification of the causal mutation is crucial for the patient's diagnosis (7).

Genetic testing in VWD has the highest importance for confirmation type 2 VWD. Fortunately, many type 2 mutations are located in exons 18 to 24 (type 2N) and in exon 28 (types 2A, 2B, and 2M), which makes DNA sequencing analysis straightforward in these cases (12). In case of decreased FVIII levels, especially in males, it is necessary to distinguish Hemophilia A from type 2N VWD. Identification which disorder is present and a type of inheritance is necessary for prediction of bleeding risk in family members and may influence the choice of treatment. Genetic analysis of the exons encoding the D´-D3 domains of *VWF* and *F8* may identify the mutation(s) and allow differential diagnosis, if necessary (7, 13).

Rare phenotype 2B VWD may result from sequential variants of either the *VWF* gene or the *GP1BA* gene encoding GP Ibα. Sequence analysis of the 5'-end of *VWF* exon 28 may identify missense mutations in the A1 domain responsible for VWD type 2B. Where absent, analysis of the exons 1–2 of the *GP1BA* gene indicates changes in the sequence corresponding to the phenotype associated with the platelet type VWD (PT-VWD). Differentiation is necessary due to the different treatment of these two diseases (7, 13).

Types 2A and 2M VWD are difficult to distinguish, based on the phenotype. Multimer analysis or genetic testing may be helpful. Genetic analysis identifies a causal mutation and may indicate which of these two subtypes is present. Only a small proportion of patients with 2A have recessive inheritance, the dominant inheritance is more common. Identification of mutations in D2 or CK domains may indicate a lower risk of inheritance for family members because mutations at these sites may be associated with recessive inheritance in type 2A (IIC). Patients with 2A rarely respond well to treatment using desmopressin, while 2M patients more frequently respond to this treatment (7, 13).

Genetic testing in VWD type 1 is less frequent because it requires a sequence analysis of the entire *VWF* gene and the causal mutation does not occur in all cases. For type 3 VWD genetic analysis may be useful in assessing the risk of future offspring being affected. If parent mutation is identified, it is possible to perform prenatal diagnosis of chorionic villus, less frequently from amniocytes or possibly preimplantation genetic diagnosis (7, 13).

## Problems associated with molecular analysis of the VWF gene

Professor Favaloro in his article (14) pointed out the main problems and the most common errors associated with genetic testing of the *VWF* gene. The first problem is the large size of the *VWF* gene and the fact that for types 1 and 3 sequence analysis of the entire gene is necessary because the mutations are localized throughout the *VWF* gene. This greatly increases the financial and time requirements. In the Slovak Republic genetic diagnostics in VWD is not funded by health insurance companies, genetic testing is not a routine part of the screening for VWD. It is mostly implemented only within the scope of scientific research projects.

Another problem is the presence of a partial pseudogene on chromosome 22, which requires special pairs of primers designed on the basis of differences between the functional gene and the pseudogene to ensure that a functional gene is actually tested (7, 14).

In recent years there is more evidence that the *VWF* gene is highly polymorphic. More than 150 polymorphisms have been described in its sequence. More than 50 of them is changing the coding sequence. This may lead to a misclassification of mutations in the *VWF* gene as polymorphisms and *vice versa*. Thus, differentiation of neutral variations in the sequence from pathological mutations is challenging, especially in type 1 VWD and in the case of rare polymorphisms (7, 14).

There are large differences in assigning of the VWD type to a certain mutation. Mutations located in a close proximity have been assigned as different types of VWD. Even mutations with the same localization but with a different amino acids exchange can lead to different types of VWD. This points to the complexity of this issue (7, 14).

Although there was a hope that genetic testing would allow identification of the genotype and phenotype correlation in all VWD patients, many patients with the same *VWF* mutation have different plasma profiles, intensity and form of bleeding manifestations. It should be noted that plasma levels of VWF are affected by many factors – blood group, hormones, pregnancy, certain medications, some diseases, stress, inflammation, physical activity. Therefore, in addition to individual genetic factors, these environmental factors need to be taken into account (7, 14).

## Future perspectives

According to large multicenter studies no causal mutation in the *VWF* gene was identified in 35% of patients with type 1 VWD. These are usually cases with a mild form of VWD. It is likely that the genetic determinants of low VWF levels are more complex and may include intronic variants and/or variants on another genetic loci. The identification of new loci that may affect VWF levels in normal individuals is the subject of several recent studies. Preliminarily, these studies suggest that some of the new variants may modify the phenotype in VWD type 1 and it is a long-term goal to incorporate these analyzes into the diagnostic algorithm for this multigenic sign (15).

Epigenetic changes may also play certain role in influencing VWF plasma levels. Circulating microRNA is at the center of attention as a new biomarker in many diseases. Studies dealing with *VWF* gene regulation have been focused on the 5'UTR region via promoter and related transcription factors, for a long time. Xiang *et al* focused on the 3'UTR region in their study and noted that expression of the *VWF* gene was also regulated in this area using miRNA. They identified that miR-24 and miR-335 regulate the human 3'UTR region of the *VWF* gene. miR-24 strongly controls VWF levels by a pleiotropic effect, including a direct binding to the *VWF* 3'UTR region. Furthermore, they observed that hyper-

glycemia induced suppression of miR-24 enhances the expression and secretion of VWF in patients with *diabetes mellitus* (16, 17).

**Our experience**

The National Centre of Thrombosis and Haemostasis in Martin is the first department in Slovakia who have already started genetic analysis of *VWF* gene in patients with VWD. The standard method used to identify causal mutations is the amplification of patient´s DNA using polymerase chain reaction and subsequent sequencing by the Sanger method. This is a time-consuming method because types 1 and 3 require sequencing of the entire *VWF* gene comprised of 52 exons.

More efficient methods of molecular biology, next generation sequencing (NGS), seem to be more effective. The principle of increased efficiency is in the massive parallelization of biochemical and measurement steps as well as in a markedly increased sequencing speed. The new methods allow simultaneous analysis of millions of sequential "readings" in one sample. Sequencing so-called gene panells (panel sequencing) is targeted to particular diseases as a set of known, clinically relevant genes are analyzed. This achieves a greater coverage depth (i.e., more readings in the desired areas) and, consequently, higher analytical sensitivity and specificity (18). NGS analysis consists of three main phases: preparation of DNA library (fragmentation of DNA into smaller fragments, up to 500-1,000 bp), amplification of clones and synthesis of complementary strand from each DNA fragment, parallel sequencing of the entire library. Each of the four free deoxynucleotides (dNTP) is usually labeled with a different fluorescent dye. After the nucleotide is inserted, the signal from each cluster is recorded, cyclically repeated until the entire library is sequenced. Candidate causal variants identified using this technique require the use of Sanger sequencing to confirm the identified mutations (7).

Our aim is to undergo our patients with VWD diagnosis for screening of mutations in *VWF* gene using the next generation sequencing for analysis of the whole *VWF* gene. We have a custom designed panel which covers exons 1–22 and 35–52 including 25 bp flanking introns on each side due to possible splice sites mutations as well as 3´and 5´ untranslated regions (UTR) of *VWF* gene. The panel does not cover sequences homologous with pseudogene sequence, exons 23–34. For this region we use classic Sanger sequencing with special pairs of primers considering the precise differences between the *VWF* genomic sequence and the pseudogene, to ensure highly specific amplification, by Corrales *et al.* (12). Sanger sequencing is also used for verification of variants which are obtained from NGS analysis. The obtained variants are compared to the reference sequence NC_000012.12 and subjected to bioinformatic analysis.

Currently we are investigating first seven patients using the above mentioned approach and we believe to have the first results soon. Our results can verify whether these methods are suitable for use as a diagnostic tool. We believe that our results will help to explain the origin of defect in *VWF* gene found in our patients. By identifying causal mutation we would like to complete VWD diagnostics in these patients using all available methods. Based on these examinations we can reliably identify the patient´s type of VWD and, thus, ensure proper management for these patients.

## CONCLUSION

Genetic testing in VWD is of the highest importance in type 2 VWD. Based on the patient's phenotype we can predict the localization of disease causing the mutations. Type 1 and 3 VWD require sequencing of the entire *VWF* gene comprised of 52 exons. The large size of the *VWF* gene together with the presence of a partial pseudogene *VWF* is one of the problems encountered in sequencing the *VWF* gene. Relatively new methods of the new generation sequencing seem to be helpful.

Many patients with VWD type 1 are free of mutation in the *VWF* gene, suggesting that other genetic variants such as mutations in the *VWF* gene may be the cause of the VWD. Therefore, further research is sought to look for other potential variants that may affect VWF plasma levels in these patients.

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