The molecular pathogenesis of type 3 von Willebrand disease in Hungary: details of the „Hungarian mutation” and alloantibody formation

Thesis of PhD dissertation

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Introduction

Von Willebrand disease (VWD) is the most common hereditary coagulation abnormality described in humans, first to describe by the Finnish internist Eric von Willebrand. It arises from a qualitative or quantitative deficiency of von Willebrand factor (VWF). VWF is a multimeric glycoprotein, produced by megakaryocytes and endothelial cells, required for platelet adhesion and to protect factor VIII (FVIII) from rapid breakdown within the blood. The absence of VWF presents with bleeding tendency affecting the primary and secondary haemostasis. There are three types of VWD described: type 1 is a quantitative defect characterized by decreased levels of normal multimeric VWF. Type 2 VWD is a qualitative deficiency of VWF with the presence of low molecular weight multimer (LMWM) units in the circulation. Type 3 is the most severe form of VWD caused by the virtual absence of VWF. Types 1 and 2 are inherited as autosomal dominant traits and type 3 is inherited as autosomal recessive. The VWF gene is located on chromosome 12, spanning 178 kilobase pairs.
and contains 52 exons. The different mutations causing VWD are very heterogeneous, and are scattered throughout the entire VWF gene.

**Aims of the study**

We set out to genotype the Hungarian type 3 population by direct sequencing. Patient selection was facilitated by a nation-wide Bleeding Diathesis Registry. Detailed laboratory analysis of the von Willebrand protein was performed, and clinical characteristics of the patients were summarized using standardized questionnaire. Bleeding scores were calculated as described previously. Detailed genealogical information was extracted through interviews with patients family members. This paper summarizes the clinical characteristics of the patients and the genetic defects. The prevalence of type 3 VWD in Hungary is **2.6 per million** (26 registered patients from 24 families)
**Methods**

24 type 3 patients from 23 unrelated families were enrolled in the study (13 females 12 males). Inclusion criteria were as follows: (1) VWF:Ag < 5 IU/dl and (2) a family history compatible with recessive inheritance. One additional patient with VWF:Ag of 6 IU/dl and a family history compatible with recessive inheritance, who carried the diagnosis of type 3 VWD, was enrolled into the study.

**Bleeding score, genealogical investigation:** A standardized questionnaire was filled out by the interviewing physician, evaluating the bleeding tendency, treatments received, and family data. Bleeding scores were calculated as described previously. Detailed genealogical information was extracted through personal and telephone interviews with patients and knowledgeable family members.

**VWF antigen and activity measurements:** VWF antigen concentrations (VWF:Ag) and VWF collagen binding activity (VWF:CBA) were determined by ELISA methods (Dako, Glostrup, Denmark; Sigma, St. Louis, MO, USA).
FVIII:C was measured by a one-stage assay (ACL-10000, Instrumental Laboratory, Lexington, USA). VWF multimers analysis: electrophoresis was carried out by using a 1.6% sodium dodecyl sulfate (SDS) agarose gel followed by electric transfer to a nitrocellulose membrane. Multimers were visualized using the chemiluminescent visualization kit from Amersham Pharmacia Biotech, Uppsala, Sweden.

**Molecular studies:** The molecular studies were carried out in the Laboratory of University Medical Centre Hamburg-Eppendorf, Department of Paediatric Hematology and Oncology, Hamburg, Germany. Primer pairs were designed for the PCR (polimerase chain reaction) amplification of the 51 coding exons and their flanking intronic regions of VWF. For exons 23 through 34, primer sequences were designed to avoid amplification of the VWF pseudogene. Direct sequencing of the PCR products were performed (ABI, Foster City, CA, USA). The breakpoints of delExon1–3 were characterized by standard gene mapping.
Breakpoint-specific PCR was designed to confirm the presence of the deletion using two primer sets contain a common reverse primer and two different forward primers. The PCR allowed the simultaneous amplification of two different products in the presence of delExon1–3 or normal allele.

Gene dosage analysis A multiplex PCR was designed using primers for the target (VWF exon 3) and reference (exon 3 and exon 9 of Rab27a on chromosome 15) sequences. The PCR products were then analyzed with denaturing high-performance liquid chromatography (Transgenomic; Omaha, IA, USA), and ratios under the two curves (VWF/reference) were calculated.

Results

Clinical characteristics: VWF:Ag level was ≤ 1 IU/dl in 21 patients, and was 1-5 IU/dl in 3 patients. Traces of low molecular weight multimers (LMWM) of VWF were present on the multimer gel of 4 patients. Of these 4 patients, 3 had VWF:Ag levels higher than 1 IU/dl. No traces of VWF were detectable on the multimer gel of 20
patients, all these patients had VWF:Ag levels ≤ 1 IU/dl. Detailed clinical information and bleeding scores could be obtained from 24 patients. The bleeding score was 12-28 (mean: 17.8) in male, and 10-32 (mean: 21.7) in female patients, representing more severe bleeding symptoms in female patients due to menorrhagia, ovarian and postpartum bleeding. Alloantibodies to VWF were present in one patient, who developed an anaphylactic reaction to the VWF concentrate (rate of inhibitor development 1/24 or 4.2%).

Molecular characteristics: 14 novel mutations were identified in 30 alleles. Five being nonsense mutations (p.Q1238X, p.Q1898X, p.Q1931X, p.S2505X, p.S2568X), 4 frameshift mutations (c.1992insC, c.3622delT, c.5315insGA, 7333delG), 3 missense (p.C35R, p.R81G, p.C623T) and 1 candidate splice site mutation (c.1730-10C>A). In addition, one partial large deletion was identified causing the loss of the 5’-end of the VWF gene. 6 previously described mutations were detected in 16 alleles, including c.2435delC, p.R1659X and p.R1853X, frequently found in several European
populations. Besides, two previously described frameshift (c2124delCT, c2269delCT) and 1 splice site (c3379+1G>A) mutation was identified. In one patient, no clear explanation for the type 3 phenotype could be found.

**DelExon1-3**: One large deletion was identified in 7 patients, 12 alleles, representing 25% of all type 3. Five patients carried the mutation in homozygous and 2 patients in compound heterozygous form. The whole deletion resulted in the loss of 35 540 bp, including VWF exons 1, 2 and 3. The 5’ breakpoint of the deletion was localized in the non-coding region between the VWF and CD9 genes, while the 3’ breakpoint was found in intron 3 of VWF. Sequence analysis of the deletion showed repetitive AluY and AluSP elements at the breakpoints. The deletion is suspected to have resulted from an illegitimate homologous recombination event between the Alu sequences. The common single-nucleotide polymorphisms (SNP) in the sequenced DNA fragments and variable tandem repeat (VNTR) in intron 40 in the patients carrying delExon1–3 favours a founder effect. The results of genealogical studies suggest that this
founder effect is at least several hundred years old. Using the deletion specific PCR, we screened 175 German, Russian and Polish VWD patients, but the delExon1–3 was not present in any of these patients. None of the delExon1-3 patients developed alloantibodies against VWF.

The frameshift mutation c.2435delC, frequently found in several European populations, was present in 5 type 3 patients. Five patients were heterozygous, and one was homozygous for this mutation. c.2435delC was represented in 12.5% of all type 3 alleles (6/48). The only patient developing an inhibitor to VWF carried the homozygous c.3622delT mutation.

Three novel missense mutations were detected, all of them in compound heterozygous form with a nonsense mutation. All of the missense mutations were localized in the coding region of the propeptide, 2 of them causing the loss of a cysteine residue, p.C35R, and p.C623T in exons 3 and 15. The third mution resulted the p.R81G amino acid change in exon 4. Traces of LMWMs could be detected on multimer
gels of the patient carrying p.C623T, while no VWF could be detected in the two other patients.

One additional patient was included in the study, because she had severe VWD with a recessive inheritance, and was previously labeled with the diagnosis of type 3 VWD. This patient had VWF:Ag 6 IU/dl and detectable LMWM on multimer gel. The VWF:Ag/VWF:CBA ratio was high (6/1). She was found to have the propeptide missense mutation p.C295S in compound heterozygous form with a null allele (c.2435delC). Given the VWF concentration above 5 IU/dl, and a multimer pattern compatible with type 2A (variant IIC) VWD, she is more appropriately classified as type 2A (variant IIC). The IIC variant is a rare subtype of type 2A, inherited as autosomal recessive traits. Previous genetic defects causing variant IIC phenotype were located in the propeptide, presumably interfering with the multimerisation process.
Conclusions

In summary, we report the genetic defects of virtually the entire type 3 population in Hungary. We performed the direct DNA sequence analysis of the Hungarian type 3 VWD population, as well detailed clinical, laboratory and genealogical data were collected. Like previously reported populations, the Hungarian cohort were found to be heterogeneous, but interestingly, we found a common new large deletion. In contrast to several previous reports, none of the patients homozygous for the large deletion developed alloantibodies to VWF. The reason for this discrepancy is not known, but it may indicate selection bias in some of the previous reports. Together delExon1-3 and c.2435delC were detected in 37.5% of all type 3 alleles, offering a rational approach to genetic testing. One patient developed an inhibitor to VWF (incidence: 4.2%, 1/24), who carried the homozygous c.3622delT mutation. In addition, we report several new mutations that may open new windows on VWF biology.
Articles related to the Ph.D. theses

1. **IF: 6,069**

2. **IF: 6,291**
   An Alu-mediated novel large deletion is the most frequent cause of type 3 von Willebrand disease in Hungary.

3. **IF: 6,069**
   Accelerated clearance alone explains ultra-large multimers in von Willebrand disease Vicenza.
Other articles

1. IF: 2,640