Pathophysiology of von Willebrand factor in bleeding and thrombosis

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PART

GENETICS OF VON WILLEBRAND DISEASE

CHAPTER 2

DIFFERENTIAL DIAGNOSIS BETWEEN TYPE 2A AND 2B VON WILLEBRAND DISEASE IN A CHILD WITH A PREVIOUSLY UNDESCRIBED *DE NOVO* MUTATION

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LETTER TO THE EDITOR

Type 2 von Willebrand disease (VWD) is due to qualitative defects of von Willebrand factor (VWF) and can be further divided into four subgroups: (i) type 2A includes variants with decreased VWF-dependent platelet adhesion and the deficiency of high molecular weight multimers (HMWM); (ii) type 2B includes variants with increased affinity of VWF for platelet glycoprotein Ibα (GPIbα) and a variable deficiency of HMWM; (iii) type 2M includes variants with decreased VWF-dependent platelet adhesion without selective deficiency of HMWM and (iv) type 2N includes variants with markedly decreased binding affinity for factor (F)VIII [1]. Patients with the most common form of type 2A [2] and type 2B [3] VWD may have similar FVIII and VWF levels (FVIII coagulant activity [FVIII:C], VWF antigen [VWF:Ag], VWF ristocetin cofactor activity [VWF:RCo], VWF collagen binding [VWF:CB]) and also the multimeric pattern may be quite similar. On the contrary, ristocetin induced platelets agglutination (RIPA) is remarkably different in type 2A and 2B VWD patients, being impaired in the former and heightened in the latter. RIPA is the most commonly used method to discriminate type 2B from other VWD types, although it suffers of limitations such as the need of a fresh blood sample and the requirement of an adequate patient platelet count [4]. Furthermore, we prefer not to perform RIPA in paediatric patients, due to the relatively large amount of required blood. There are other assays which are able to discriminate type 2B VWD using smaller amount of blood such as: (i) the measurement of ristocetin-induced binding of VWF to platelets by flow cytofluorimetry, performed using autologous platelets [5] and (ii) the whole blood ristocetin-activated platelet impedance aggregometry [6], although these assays are not currently available in our laboratory. Molecular analysis is another valid alternative to RIPA assay even if it might be occasionally inconclusive as in the case herein discussed.

Flood *et al.*, [7] developed a ristocetin independent ELISA method using a recombinant gain-of-function GPIbα [7] to evaluate the platelet-dependent VWF activity (VWF:GPIbM) [8] and claimed to be able to discriminate type 2B from the other type 2 VWD patients using this assay [7]. Recently, we proposed a modified version of the Flood's ELISA as an alternative to RIPA to differentiate type 2B from the other VWD types, showing that the VWF:GPIbM/VWF:RCo ratio distinguishes most type 2B VWD patients from non-type 2B VWD. Indeed, type 2B VWD patients showed much higher VWF:GPIbM/VWF:RCo ratios than healthy subjects and other VWD patients. Furthermore, among type 2B patients, the increase in this ratio is proportional to the loss of HMWM [9].

With this background, we describe the practical use of the VWF:GPIbM/VWF:RCo ratio as an alternative to RIPA to perform the differential diagnosis between type 2A and 2B VWD in a paediatric patient with a previously undescribed *de novo* mutation. All subjects were aware of the study and gave their informed consent, according to the Declaration of Helsinki. The bleeding history of the proband, collected using the International Society On Thrombosis And Haemostasis-Bleeding Assessment Tool (ISTH-BAT) [10] and obtaining a score of 5, was characterized by easy bruising, prolonged bleeding after minor wounds

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and recurrent epistaxis that in one instance required cauterization. The last event of epistaxis was followed by hemorrhagic shock which required blood transfusion and replacement therapy with a FVIII-VWF plasma-derived concentrate in association with tranexamic acid. The biochemical analysis of the 2 years old girl showed a platelet count of 323.000/mm^3 and FVIII:C of 56 IU/dL, but reduced VWF levels (VWF:Ag 33 IU/dL, VWF:RCo 6 IU/dL, VWF:CB 3 IU/dL) [11]. Multimeric analysis, performed by electrophoresis using 1.2% HGT agarose gel in presence of 0.1% SDS [12], showed the lack of high/intermediate MWM in the proband's plasma (**Figure 1A**).

The proband's parents had normal FVIII and VWF levels (FVIII:C 156 IU/dL, VWF:Ag 128 IU/dL and VWF:RCo 115 IU/dL for the mother and FVIII:C 130 IU/dL, VWF:Ag 119 IU/dL and VWF:RCo 110 IU/dL for the father).

The lack of high/intermediate MWM and the markedly reduced VWF:CB (3 IU/dL; normal range 45-174 IU/dL [11]) excluded a diagnosis of type 2M, but led to a possible diagnosis of type 2A or 2B VWD. The normal platelet count of the proband (323.000/mm^3) suggested a type 2A VWD diagnosis, because most type 2B patients with a severe loss of high/intermediate MWM are thrombocytopenic.

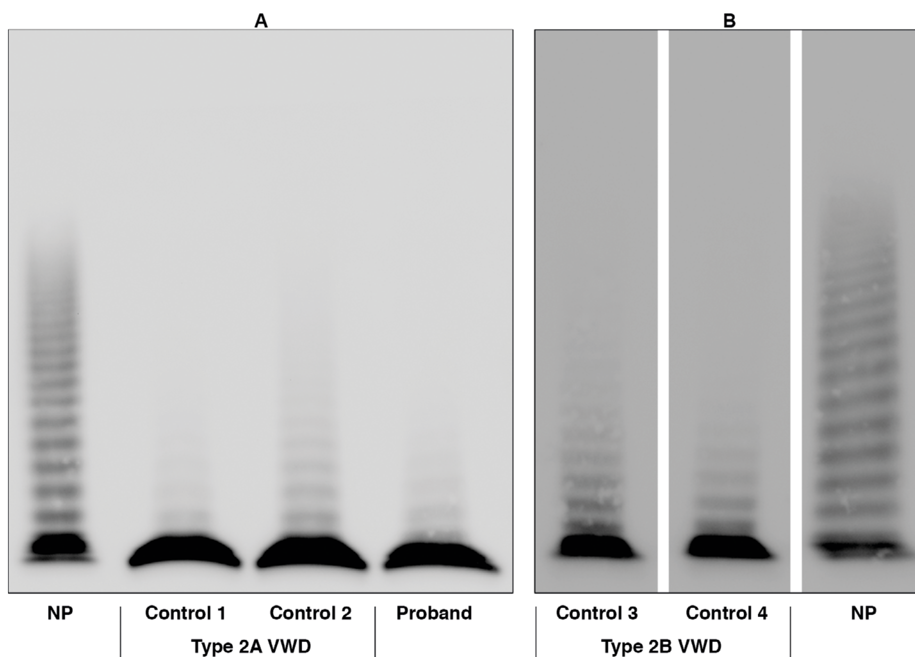

Figure 1. Multimeric analysis of proband and type 2A and 2B VWD patients used as controls. Multimeric structure of plasma von Willebrand factor (VWF) visualized in a non-reducing agarose gel (1.2% HGT agarose/0.1% SDS). The plasma VWF of the proband and those of type 2A (**A**) and 2B VWD (**B**) patients used for comparison showed a comparable multimeric pattern with the lack of high and intermediate molecular weight multimers. Lanes from the same gel are delimited by a black line.

Sanger sequencing of *VWF* exon 28 was performed for the proband in order to investigate the presence of a type 2 VWD mutation in both the A1 and the A2 domains [13] and a previously undescribed heterozygous in-frame deletion (c.[4606_4611delCACGTC];[=], p.[H1536_V1537del];[=]) was identified in addition to the already reported polymorphisms c.4141A>G (p.Thr1381Ala) and c.4641T>C (p.Thr1457Thr), both in homozygosis (reference sequence NM_000552.4). Then, the candidate mutation (p.H1536_V1537del) found in the proband was also searched in both parents without success. Short tandem repeats (STR)I and II were analysed by PCR in the proband and her parents using labelled oligonucleotides as previously described [14] to confirm the *de novo* origin of the mutation. The proband showed the same alleles (STRI-II 101-162 bp and STRI-II 121-170 bp) and also identified in her mother (STRI-II 101-162 bp; STRI-II 105-158 bp) or father (STRI-II 121-170 bp; STRI-II 125-182 bp).

Therefore, we could conclude that p.H1536_V1537del is a *de novo* mutation, which arose either in the proband herself at the early stages of embryogenesis or in the germ line cells of one of her parents. *De novo* mutations are rarely reported for VWD and their frequency is not precisely estimated [15], although Shen *et al.* [16] have recently described 4 *de novo* mutations in 15 families affected by type 2 VWD . Based on the STRI-II analysis, the proband has inherited the *VWF* alleles from her unaffected parents. Therefore, the *de novo* mutation identified is likely to be the disease-causing mutation, as also supported by the *in silico* evaluation performed using PROVEAN (-6.5; cut-off of -2.5) [17], SIFT-indel (0.894; max= 1) [18] and MutationTaster (damaging mutation) [19].

In this case, molecular analysis did not help us to distinguish between type 2A or type 2B VWD, because this mutation has never been reported before. Then, we carried out the VWF:GPIbM assay. The plasma samples of two type 2A and two type 2B VWD patients, characterized by the lack of the high/intermediate MWM and by plasma VWF levels similar to those of the proband, were used as controls (**Figure 1 A,B**). Each plasma sample was determined twice at two different dilutions.

The proband had a VWF:GPIbM/VWF:RCo ratio of 1, similar to those of the type 2A VWD controls but lower than those of type 2B VWD (**Table 1**).

This allowed us to exclude a type 2B gain-of-function effect for this mutation, thus obtaining the type 2A VWD diagnosis. A limitation of this approach is that the VWF:GPIbM/VWF:RCo ratio is inadequate to evaluate a possible type 2A patient with a VWF:RCo< 6 (IU/dL). In this case, the comparisons between VWF:GPIbM and VWF:RCo versus the corresponding VWF:Ag values have to be considered. Indeed, in type 2A VWD patients, the discrepancy between VWF activity and VWF:Ag is present when the activity is measured with both VWF:GPIbM and VWF:RCo assays, whereas in type 2B VWD patients this discrepancy is absent when the VWF:GPIbM assay is performed (**Table 1**).

In conclusion, this is a rare case of a previously undescribed *de novo VWF* mutation causing type 2 VWD. Both the platelet count and the localization of the p.H1536_V1537del mutation in the A2 domain suggested the type 2A VWD phenotype, although a firm diagnosis was obtained only by evaluating the VWF:GPIbM/VWF:RCo ratio.

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Table 1. Biochemical and molecular data of proband and type 2A and 2B VWD patients used as controls

Patient	Type	Amino acid substitution	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:GPIbM *(IU/dL)*	VWF:GPIbM/ VWF:RCo
Proband	2A or 2B	p.[H1536_V1537del];[=]	33	6	6	1
Control 1	2A	p.[S1506L];[=]	24	8	10	1.3
Control 2	2A	p.[R1597Q];[=]	39	7	9	1.3
Control 3	2B	p.[R1306W];[=]	35	<6 [a]	42	8.4
Control 4	2B	p.[V1316M];[=]	35	8	34	4.5

Mutations are reported following the guidelines of the Human Genome Variation Society (http://varnomen.hgvs.org/; accessed on April 2018). Type 2A and type 2B VWD plasma samples with VWF levels and multimeric patterns comparable to those of the proband were used as controls. VWF:Ag, von Willebrand factor antigen; VWF:GPIbM, VWF gain-of-function mutant GPIb-binding assay; VWF:RCo, VWF ristocetin cofactor activity. [a] Because the lower detection limit of the aggregometric VWF:RCo test is 6 IU/dL, a value of 5 was considered in the ratio calculation.

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