Pathophysiology of von Willebrand factor in bleeding and thrombosis

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CHAPTER 3

VON WILLEBRAND DISEASE TYPE 1 MUTATION p.Arg1379Cys AND THE VARIANT p.Ala1377Val SYNERGISTICALLY DETERMINE A 2M PHENOTYPE IN FOUR ITALIAN PATIENTS

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ABSTRACT

Introduction

We characterized five patients affected with von Willebrand disease (VWD) carrying the p.Arg1379Cys mutation. One was diagnosed as VWD type 1 and four as type 2M. The 2M patients also have the variant p.Ala1377Val *in cis* with p.Arg1379Cys.

Aim

To evaluate the role of p.Ala1377Val and p.Arg1379Cys von Willebrand factor (VWF) variants to explain patients' phenotype.

Methods

Conventional phenotype tests were used to evaluate patients' plasma and platelets. Direct sequence analysis of exon 28 was carried out. The allele frequency of p.Ala1377Val was evaluated using online database. pcDNA3.1-VWF-WT and mutant (A1377V, R1379C and A1377V-R1379C) expression vectors were transiently transfected in HEK293 cells. The capacity of WT and mutant recombinant (r)VWF (along with patients' plasma VWF) to bind glycoprotein Iba(GpIbα) were evaluated, using two ELISA assays. One with a wild-type (WT) recombinant (r)GpIbα at increasing ristocetin concentrations (from 0 to 1.50 mg/mL) and the other with a gain-of-function mutant rGpIbα (VWF:GPIbM).

Results

The substitution c.4130C>T (p.Ala1377Val) was reported as rare variant in online databases. At 0.25 mg/mL of ristocetin, WT, A1377V and R1379C showed 6, 7.5 and 12-fold increased binding to rGpIbα, respectively. A1377V-R1379C rVWF showed no increased binding to rGpIbα at the same ristocetin concentration and reached the highest binding, of only 3-fold increased, at 1.50 mg/mL of ristocetin. The VWF:GPIbM showed strongly reduced values for the A1377V-R1379C rVWF and the 2Mpatients' plasma.

Conclusion

Our study showed that the presence of both p.Ala1377Val and p.Arg1379Cys mutations (synergistic effect) abolishes the binding of rVWF to rGpIbα, explaining patients' 2M phenotype.

INTRODUCTION

von Willebrand disease (VWD) is a common bleeding disorder due to quantitative and/or qualitative defects of von Willebrand factor (VWF) [1]. This multimeric glycoprotein plays an important role in primary haemostasis, promoting platelet adhesion at sites of vascular injury and in secondary haemostasis serving as a carrier protein for factor VIII (FVIII) [2-3]. A revised classification distinguishes quantitative defects of VWF (types 1 and 3) from qualitative defects (types 2A, 2B, 2M, and 2N) [4]. VWD type 1 and type 3 include partial quantitative deficiency and virtually complete deficiency of VWF, respectively. VWD type 2A includes variants with decreased VWF-dependent platelet adhesion due to a loss of VWF high and intermediate molecular-weight multimers (MWM). VWD type 2B is characterized by an increased affinity for platelet glycoprotein Ibα (GpIbα). VWD type 2M includes variants with decreased VWF-dependent platelet adhesion, without a selective deficiency of high-MWM [4]. VWD type 2N is characterised by a markedly decreased binding capacity of VWF to FVIII [4-5]. Molecular analysis is a valid effort to characterise patients' phenotype in most type 2 VWF variants, caused by mutations in exon 28 (2A, 2B, and 2M) or in exons 18 to 24 (2N) [4].

In this study, we characterized five unrelated patients with VWD using biochemical and molecular analysis. Direct sequencing of exon 28 revealed the presence of p.Arg1379Cys mutation previously reported to cause VWD type 1 [6-8], in all five patients. However, only one of these patients was classified as type 1, whereas the other four were diagnosed as type 2M. The four type 2M patients had the additional substitution p.Ala1377Val *in cis* with p.Arg1379Cys previously reported as polymorphism on the VWF variants database (VWFdb; http://www.vwf.group.shef.ac.uk/vwd.html; accessed August 2016), so its role as disease-causing mutation was initially underestimated. We investigated the role of p.Ala1377Val and p.Arg1379Cys variants, in order to establish their influence in the binding of VWF to GpIbα with the goal to explain the patients' phenotype.

METHODS

Patients

All five patients were aware of the experimental nature of this study and gave informed consent, according to the declaration of Helsinki (1964). Patients bleeding history was collected using the published bleeding severity score (BSS) [9].

Blood sampling and laboratory methods

Twenty millilitres of blood was drawn in 3.13% sodium citrate (9:1 v/v) and 5 mM EDTA (9:1 v/v). FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) were evaluated as previously described [10]. Platelets were isolated using a density gradient and lysed using Triton X-100 [11]. Ristocetin-induced platelet agglutination (RIPA) was carried out in platelet-rich plasma (PRP) using different

ristocetin concentrations [12]. VWF collagen binding activity (VWF:CB) of patients' plasma was evaluated using collagen type I (95% type I and 5% type III; Nycomed-Horm), as previously reported [10]. Plasma VWF multimeric analysis was performed by gel electrophoresis [13] under non reducing conditions in low (1.2% HGT agarose/0.1% SDS) and intermediate resolution (1.6% LGT agarose/0.1% SDS) gels. Determination of VWF propeptide (VWFpp) was performed by ELISA assay using the anti-human VWF propeptide antibodies (Sanquin, Amsterdam, The Netherlands).

Nomenclature

The mutations are reported following the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/recs.html; accessed August 2016).

VWF analysis

Genomic DNA was extracted from peripheral blood using standard methods. The polymerase chain reaction (PCR) was performed to amplify the *VWF* exon 28 of patients as previously reported [14] to investigate the presence of mutations in the VWF A1 domain. Oligonucleotides and PCR conditions are available on request. Short tandem repeats (STR) I and II [15] were analysed by PCR using labelled oligonucleotides as previously described [16]. Molecular analyses of patients' relatives were performed to establish the *cis/trans* state of the two mutations for patients II, IV and V and to determine the STR I and II polymorphisms linked to patients' mutated allele. As no family members of patient III were available, to evaluate the *cis/trans* state of the two variants in patient III, a PCR fragment of a portion of exon 28 (939 base pairs) was purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), cloned into the pCR®XL-TOPO® vector (TOPO® XL PCR Cloning Kit; Invitrogen, Carlsbad, CA, USA) and sequenced.

The evaluation of p.Ala1377Val allele frequency among population has been performed using Exome Variant Server (http://evs.gs.washington.edu/EVS) and 1000 Genomes database (http://www.1000genomes.org), both accessed on August 2016 [17].

In silico evaluation

The potential effect of p.Ala1377Val and p.Arg1379Cys on the destabilization of the A1 domain, was analysed using the crystal structure of the wild-type (WT) A1 domain (1AUQ.pdb; Protein Data Bank, http://www.rcsb.org/pdb/search/searchSequence.do) and the I-mutant 3.0 tool (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) at default mode, both accessed on August 2016 [18]. PYMOL software (DeLano Scientific, San Carlos, California, USA) was used to mutagenized the 1AUQ.pdb and to analyse the impact of both variants on the secondary structure of the A1 domain in comparison with the WT.

Plasmid construction

Mutant expression vectors pcDNA3.1-VWF-A1377V, pcDNA3.1-VWF-R1379C, and pcDNA3.1-VWF-A1377V-R1379C were generated by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit; Agilent Technologies, La Jolla, CA, USA), using as template the pcDNA3.1-VWF-WT vector [19] and specifically designed oligonucleotides.

Transfection experiments

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle's medium (DMEM):F12 supplemented as previously reported [19]. Cells at 80-90% of confluence were transiently transfected with 8 µg of plasmid DNA using jetPEI™ (PolyPlus-transfection, Euroclone, Pero, Italy). For WT and mutant co-transfections (hybrids) 4 µg of each vector was used. Cell media were changed before transfection and collected after 72 h. Cells were harvested and lysed as previously described [20]. WT, mutant and hybrid recombinant (r)VWF in conditioned media and cell lysates were quantified using an ELISA assay. Cell media were concentrated (10X v/v), using Aquacide II (Calbiochem, La Jolla, CA, USA). Concentrated samples were evaluated again for VWF:Ag, then evaluated for the multimeric pattern.

rGpIbα binding

The binding capacity of rVWF and patients' plasma VWF to the rGpIbα was evaluated using an ELISA assay [20] at different ristocetin concentrations (Mascia Brunelli S.p.A., Milano, Italy). rGpIbα (amino acid residues 1–305) was obtained in HEK293 cells as previously described [21]. A microtitre plate was coated with 5 µg/mL of anti-GpIbα monoclonal antibody 2D4 (Laboratory for Thrombosis Research, Interdisciplinary Research Facility Life Sciences, KU Leuven, Belgium), diluted in PBS and incubated overnight (O.N.) at 4°C. Then 100 µL of rGpIbα (1 µg/mL) was added and incubated O.N. at 4°C. Concentrated conditioned media containing rVWF, were normalized in order to allow comparisons at similar VWF:Ag levels. Similarly, the normal pooled plasma used as reference and patients' plasma were normalized for the VWF:Ag.

Fifty microlitres of each sample was seeded in duplicate for each ristocetin concentration and added with 50 µl of ristocetin (0, 0.12, 0.25, 0.50, 0.75, 1.00 and 1.50 mg/mL, final wells' concentration) in PBS. The wells were washed and bound VWF was detected with a polyclonal anti-VWF antibody conjugated with horseradish peroxidase (HRP) (DakoCytomation ApS, Glostrup, Denmark), through a colorimetric reaction. The absorbance was measured at 492 nm [20].

Gain-of-function mutant GpIbα binding (VWF:GPIbM) assay

The platelet-dependent VWF activity of rVWF and patients' plasma VWF was evaluated using a gain-of-function rGpIbα protein from amino acid residues 1–305, containing

the mutations p.Gly233Val and p.Met239Val as previously described [22]. In case of rVWF, the assay was performed on the concentrated conditioned media and the results were reported as VWF:GPIbM/VWF:Ag ratio.

Statistical analysis

The comparison between WT, mutant and hybrid rVWF levels in conditioned media and cell lysates was made using a one-way ANOVA with Bonferroni post-hoc analysis if necessary. The binding capacity of rVWF and patients' plasma VWF to the WT rGpIbα was evaluated using a two-way ANOVA with Bonferroni post-hoc analysis if necessary. P values< 0.05 were considered statistically significant.

RESULTS

Phenotypic results

Patients' laboratory data and BSS are reported in **Table 1.** Patient I showed nearly normal plasma VWF:Ag and VWF:RCo levels, whereas patient II had a nearly normal VWF:Ag value but markedly reduced VWF:RCo (<6 IU/dL). Patients III-V showed reduced VWF levels, with a VWF:RCo/VWF:Ag ratio< 0.6. The VWF:CB/VWF:Ag ratio was normal for all patients (≥ 0.6).

Multimeric analysis of patients' plasma at low resolution showed a normal pattern in patient I and a slightly loss of HMWM with smear, in patients II-V, although at different extent (**Figure 1**; upper panel). The intermediate resolution gel showed decreased or slightly decreased satellite bands of the triplet structure (i.e., diminished proteolysis) in

Table 1. Phenotype and genotype data of the five patients

Pt	ABO	BSS	FVIII:C *(IU/dL)*	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:CB *(IU/dL)*	VWF:RCo/ VWF:Ag [†]	VWF:CB/ VWF:Ag	VWFpp/ VWF:Ag
I	non-O	2	68	46	43	50	0.90	1.1	1.46
II	O	n.d.	53	38	< 6 [A]	27	0.08	0.7	2.6
III	O	3	33	17	< 6 [A]	12	0.18	0.7	3.8
IV [‡]	non-O	12	37	28	11	17	0.39	0.6	2.8
V	non-O	3	36	24	7	16	0.29	0.7	2.9
NR	-	-1 to 3	50-150	40-169 [§] 55-165 [¶]	41-160 [§] 53-168 [¶]	45-170 [§] 56-174 [¶]	> 0.6	> 0.6	0.5-1.5

Pt, patient; ABO, blood-group system; BSS, Bleeding Severity Score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:CB, von Willebrand factor collagen-binding activity; VWFpp, von Willebrand factor propeptide; RIPA, ristocetin-induced platelet agglutination. NR, normal range; n.a., not applicable; n.d., not determined. [†] A VWF:RCo of 3 IU/dL was considered, in those cases with

patients II-V (**Figure 1**; lower panel). RIPA was within the normal range (0.7 mg/mL; NR; 0.7-1.2 mg/mL) in patient I, slightly decreased in patient II (1.4 mg/mL) and strongly reduced (> 2.0 mg/mL) for patients III-V. The VWFpp/VWF:Ag ratio showed a clear VWF increased clearance in patients II-V. Platelet VWF levels were normal in patient I and markedly reduced in patients II-V.

VWF analysis

Molecular data of the patients are reported in **Table 1**. Direct sequencing of exon 28 led to the identification of the mutations c.4130C>T (p.Ala1377Val) and c.4135C>T (p.Arg1379Cys) in all subjects, but patient I in whom only p.Arg1379Cys was identified. Relatives' molecular analysis of patients II, IV and V showed that p.Ala1377Val and p.Arg1379Cys were *in cis*. Sanger sequencing of the cloned exon 28 PCR-fragment showed that p.Ala1377Val and p.Arg1379Cys were *in cis* also in patient III.

STR I and STR II analysis of patients II, IV, V and their relatives showed that the mutated allele (p.Ala1377Val-Arg1379Cys) was associated with the same STR I (101 bp) and STR II (170 bp). For patient III, STR I were 101 and 117 bp, whereas STR II were 162 and 166 bp.

Patient II showed the additional nucleotide substitutions c.3797C>T (p.Pro1266Leu) and c.3835G>A (p.Val1279Ile), both *in trans* with p.Ala1377Val and p.Arg1379Cys.

The minor allele frequency (MAF) of p.Ala1377Val (rs141211612) reported on the Exome Variant Server and 1000 Genomes database was <0.01 and <0.02, respectively.

RIPA *(mg/mL)*	Platelet VWF:Ag *(IU/10^9 platelets)*	Platelet VWF:RCo *(IU/10^9 platelets)*	Nucleotide Change	Amino Acid Change
0.7	0.32	0.35	c.[4135C>T];[=]	p.[Arg1379Cys];[=]
1.4	0.09	< 0.06	c.[4130C>T;4135C>T];[3797C>T;3835G>A]	p.[Ala1377Val;Arg1379Cys];[Pro1266Leu;Val1279Ile]
> 2	0.10	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
> 2	0.13	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
> 2	0.13	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
0.7-1.2	0.22-0.60	0.15-0.58	n.a.	n.a.

a VWF:RCo< 6 IU/dL, to perform the calculation of VWF:RCo/VWF:Ag ratio. Values are shown as a mean of three measurements in three different samples, with exception of patient IV who has been tested only once ‡ as sample was not available. Patients' platelets were isolated only once. § Range values of normal individuals with blood group O; ¶ range values of normal individuals with blood group non-O.

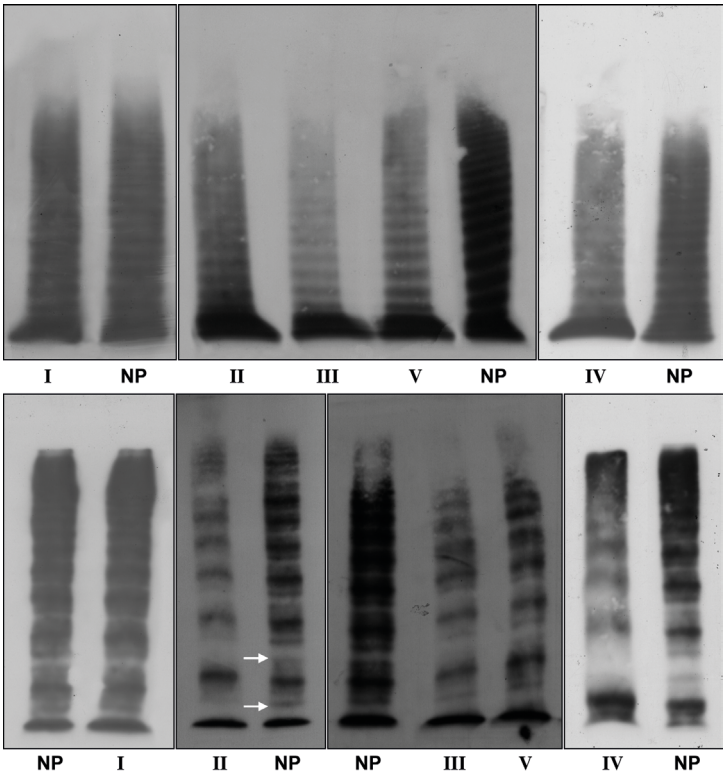

Figure 1. Multimeric analysis of patients' plasma VWF. Upper panel: Multimeric structure of plasma von Willebrand factor (VWF) visualized in a non-reducing low-resolution gel (1.2% HGT agarose/0.1% SDS) to highlight the high molecular weight multimers. All patients showed a full set of multimers, which was smeared in case of patients II-V. NP, normal plasma. Lower panel: Multimeric structure of plasma VWF visualized in non-reducing intermediate resolution gel (1.6% LGT agarose/0.1% SDS), to highlight the triplet structure. NP VWF showed the typical triplet structure and the satellite bands are indicated by the arrows. Patient I VWF showed a normal triplet structure, whereas it was decreased or slightly decreased in patients II-V. Lanes from the same gel are delimited by a black line.

Binding of patients' plasma VWF to rGpIbα

The results are reported in **Figure 2**. For each sample, the points shown in **Figure 2** correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. The plasma VWF of patient I, showed a binding to rGpIbα similar to that of the pooled plasma at each ristocetin concentration ($P > 0.05$). The plasma VWF of patients II-V showed a reduced binding to rGpIbα than the pooled plasma (1-fold vs. 2-fold, respectively; $P > 0.05$) at 0.25 mg/mL of ristocetin. The binding of patients II-V plasma VWF to rGpIbα at 0.50 mg/mL of ristocetin was reduced, although at different extent (from 2-fold to 4-fold increased vs. 5-fold increased of normal pooled plasma).

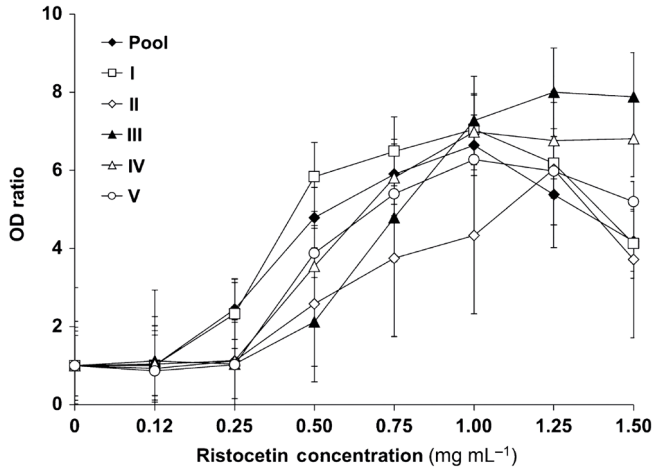

Figure 2. Binding of patients' plasma VWF to recombinant glycoprotein Ibα (rGpIbα). rGpIbα was immobilized on microtitre plates using a specific antibody. The normal pooled plasma used as reference and the patients' plasma samples were incubated in presence of increasing concentration of ristocetin (0, 0.12, 0.25, 0.50, 1.00, 1.25 and 1.50 mg/mL). For each sample, the points shown in the figure correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. Values are plotted as the mean ± SE of two independent determinations. Pooled plasma (black diamond), patient I (square), patient II (diamond), patient III (black triangle), patient IV (triangle) and patient V (circle).

VWF:GPIbM assay performed on patients' plasma

The results are reported in **Table 2**. The VWF:GPIbM values were in line with VWF:RCo values for patients I, III, IV, and V and slightly increased for patient II (**Table 2**).

In silico evaluation

I-Mutant 3.0 predicted the decrease of A1 domain stability for both mutations with a ΔΔG of -0.91 for p.Ala1377Val and -1.36 for p.Arg1379Cys.

PYMOL showed that the substitution of the Ala1377 residue with a valine does not alter the formation of hydrogen bond with the Arg1374 residue and water. The substitution of the Arg1379 residue with a cysteine determine the loss of the hydrogen bonds with Lys1407, Lys1408 and water, whereas the hydrogen bond with Ile1410 was not disrupted.

Characterization of rVWF

rVWF in conditioned media and cell lysates were quantified as VWF:Ag, using an ELISA assay. The amount of mutant and hybrid rVWF (WT/mutants co-transfections) was expressed as a percentage of the WT rVWF referred as 100% ±SEM (**Table 3**). The amount of secreted A1377V and A1377V-R1379C rVWF was decreased to (45±10%) and (42±9%) respectively, whereas the amount of R1379C and hybrids rVWF was similar to that of WT. The amount of rVWF in cell lysates were decreased for A1377V (52±10%),

Table 2. VWF:GPIbM assay performed on patients' plasma VWF

Patient	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:GPIbM *(IU/dL)*
I	50	37	34.97
II	30	< 6	11.14
III	14	< 6	3.00
IV	19	< 6	4.25
V	26	7	6.73
NR	40-169 † 55-165 ‡	41-160 † 53-168 ‡	n.d.

Pt, patient; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GpIbα binding; NR, normal range. These assays have been performed on new patients' plasma samples. † Range values of normal individuals with blood group O; ‡ range values of normal individuals with blood group non-O; n.d., not determined.

Table 3. *In vitro* expression study of recombinant von Willebrand factor

rVWF	VWF:Ag Conditioned media (%)	VWF:Ag Cell lysates (%)
WT	100 %	100 %
A1377V	45±10 %	52±10 %
R1379C	73±18 %	68±22 %
A1377V-R1379C	42±9 %	93±11 %
A1377V/WT	75±26 %	85±13 %
R1379C/WT	80±23 %	87±29 %
A1377V-R1379C/WT	95±25 %	111±11 %

rVWF, recombinant von Willebrand factor; WT, Wild-type. VWF:Ag, von Willebrand factor antigen; The amount of mutant and hybrid rVWF (WT/mutants) were expressed as a percentage of the WT referred to 100% (± SEM).

nearly normal for R1379C (68±22%) and normal for A1377V-R1379C and hybrids rVWF. All comparison made by one-way ANOVA did not show a significant difference (P> 0.05).

All rVWF showed a full set of multimers, which was particularly smeared for A1377V-R1379C rVWF (**Figure 3**).

The rVWF binding to rGpIbα at different ristocetin concentrations was calculated as performed for patients' plasma samples and reported in **Figure 4** (upper panel). WT and A1377V rVWF showed 6 and 7.5-fold increased binding to rGpIbα (P> 0.05), respectively, at 0.25 mg/mL of ristocetin. The binding capacity of R1379C rVWF to rGpIbα was higher than that of the WT with a 12-fold vs. 6-fold increased (P> 0.05) at 0.25 mg/mL. The R1379C rVWF reached the plateau at 0.25 mg/mL, whereas WT and A1377V rVWF at 0.50 mg/mL of ristocetin. The binding capacity of A1377V-R1379C rVWF was

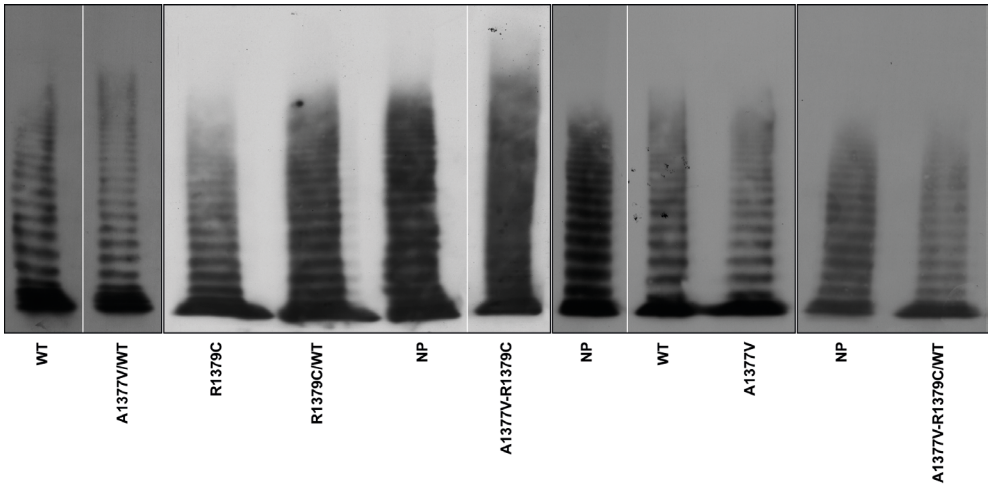

Figure 3. Multimeric structure of recombinant von Willebrand factor (rVWF) expressed in human embryonic kidney 293 cells. rVWF from conditioned media underwent electrophoresis on a 1.2% HGT agarose/0.1% SDS gel under non-reducing conditions. NP, normal plasma, WT, wild-type. Lanes from the same gel are delimited by a black line. Irrelevant lanes have been cut off (white spaces).

markedly reduced ($P < 0.05$ in the Bonferroni post-hoc analysis), showing no increasing values using ristocetin concentration varying from 0.12 to 0.75 mg/mL and reaching its highest binding capacity (only 3-fold increased) at 1.50 mg/mL. Subsequently, we analysed the A1377V-R1379C/WT rVWF binding to rGpIbα in comparison with WT and mutant A1377V-R1379C rVWF (**Figure 4**; lower panel). The hybrid rVWF showed only a slightly reduced binding capacity to rGpIbα in comparison with WT (7.6-fold vs. 10.6-fold, respectively; $P > 0.05$), at of 1.50 mg/mL of ristocetin.

The platelet-dependent VWF activity of rVWF evaluated using the VWF:GPIbM assay was reported as VWF:GPIbM/VWF:Ag ratio. The ratio was strongly reduced only for the double-mutant A1377V-R1379C rVWF (0.10) and nearly normal for the hybrid A1377V-R1379C/WT rVWF (0.86 vs. 1 of the WT). The A1377V, R1379C, A1377V/WT and R1379C/WT rVWF showed a VWF:GPIbM/VWF:Ag ratio similar to that of the WT (0.89, 0.75, 0.79, 1.00, respectively).

DISCUSSION

In this study, we described five patients carrying p.Arg1379Cys substitution already reported as VWD type 1 mutation in three different studies [6-8]. However, only one patient was diagnosed as VWD type 1. The other four patients, who also had the p.Ala1377Val variant *in cis* with p.Arg1379Cys showed a 2M phenotype.

At time of diagnosis, we investigated patient I for a type 2B mutation, due to the RIPA value (0.7 mg/mL), which was enhanced considering the VWF:RCo value (43 IU/dL). Unexpectedly, the p.Arg1379Cys mutation was identified. Only one of the previously

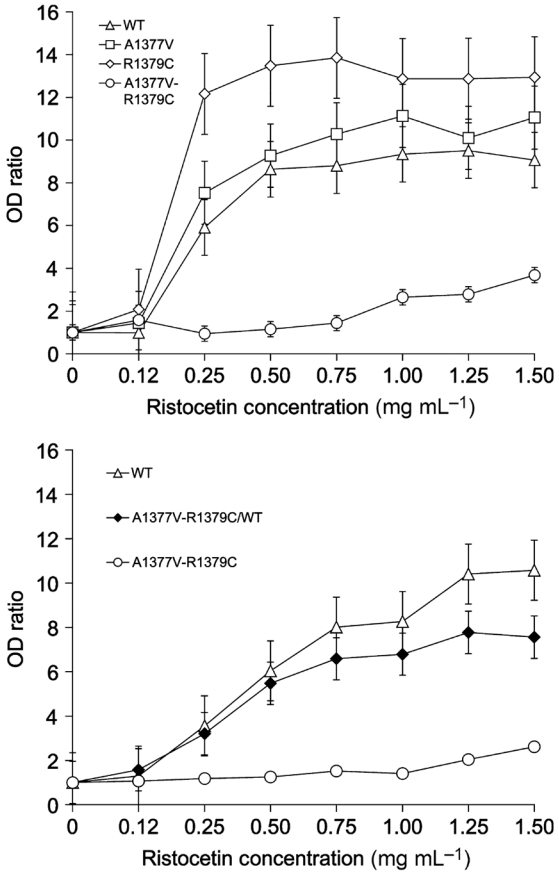

Figure 4. Binding of recombinant von Willebrand factors (rVWF) to recombinant glycoprotein Ibα (rGpIbα). rGpIbα was immobilized on microtitre plates using a specific antibody. Wild-type (WT), mutant (A1377V, R1379C, A1377V-R1379C) and hybrid A1377V-R1379C/WT rVWF were incubated in presence of increasing concentration of ristocetin (0, 0.12, 0.25, 0.50, 1.00, 1.25 and 1.50 mg/mL). For each rVWF, the points shown in the figure correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. Values are plotted as the mean ± SE of two independent determinations. WT rVWF (triangle), A1377V rVWF (square), R1379C rVWF (diamond), A1377V-R1379C rVWF (circle) and A1377V-R1379C/WT rVWF (black diamond).

reported patients carrying p.Arg1379Cys [6] showed RIPA values similar to those of patient I. Conversely, the other two studies were not able to confirm or exclude the diagnosis of mild type 2B for patients with p.Arg1379Cys [7-8]. Therefore, we confirmed in patient I the diagnosis of VWD type 1 in agreement also with *Castaman et al.*, who reported the efficacious use of DDAVP treatment in a VWD type 1 patient carrier of p.Arg1379Cys [23].

The four 2M patients showed mild bleeding symptoms and they have been treated with desmopressin in case of minor surgeries or delivery. Patient V has been treated

once with the FVIII/VWF concentrates without bleeding complications. These patients and their affected family members (**Appendix S1**) showed similar biochemical parameters, which include a strongly reduced VWF:RCo in the presence of a nearly normal multimeric pattern. These patients showed a clearly increased VWFpp/VWF:Ag ratio, as already described for other type 2M patients [24]. Interestingly, patient II showed a markedly reduced VWF:RCo (< 6 IU/dL), but only a slightly decreased RIPA (1.4 mg/mL), probably explained by the presence of the 2B New York variant (p.Pro1266Leu) [25,26] that mitigates in RIPA assay the 2M phenotype.

Initially, the pathogenetic role of p.Ala1377Val in the 2M patients was underestimated and reported as polymorphism on the VWFdb. The search on the Exome Variant Server and 1000 Genomes database showed that p.Ala1377Val was a rare variant (MAF< 1%) generally found in individuals with African ethnicity.

The finding that patients II, IV and V showed the same STR I and II indicates a possible common ancestor, explaining the presence of both substitutions in these four unrelated patients. Then, we found that the p.Ala1377Val variant has already been reported by *Millar et al.* in two patients, who showed normal VWF:Ag levels, VWF:RCo/VWF:Ag ratios (1.0 and 1.1) and multimers [27], whereas Longsdon *et al.* showed the association of p.Ala1377Val with lower VWF levels in African-Americans individuals in a burden test [28]. These data, led us to exclude that p.Ala1377Val alone was responsible for the 2M phenotype of our patients and suggested a combined effect of p.Ala1377Val and p.Arg1379Cys mutations.

The possible impact of p.Ala1377Val and p.Arg1379Cys on the VWF A1 domain structure was performed using *in silico* tools. Both variants have been predicted to decrease the A1 domain $\Delta\Delta G<0$, whereas only p.Arg1379Cys seemed to alter the secondary structure of the A1 domain by the loss of hydrogen bonds with the nearby residues (Lys1407, Lys1408) and water. We hypothesized that the presence p.Ala1377Val and p.Arg1379Cys, may cause the destabilization of the A1 domain resulting in a reduced capacity of the double-mutant VWF to bind GpIbα.

To confirm this hypothesis, we performed the *in vitro* expression study. Transfections of mutant constructs alone, showed a reduced protein secretion for A1377V rVWF and A1377V-R1379C rVWF, whereas R1379C rVWF showed antigen values similar to the WT rVWF. The hybrids, showed antigen values similar to the WT rVWF for each variant. The results of secreted R1379C/WT rVWF were consistent with the slightly reduced VWF levels of patient I, whereas those of A1377V/WT rVWF were in line with VWF:Ag levels of the patients described by Millar *et al*. [27]. There was a discrepancy between *in vitro* results of double mutant hybrid A1377V-R1379C/WT rVWF, which mimicked patients' phenotype and the reduced patients' plasma values. This was probably due to *in vitro* over-expression of transfected vectors, the difficulty to reproduce patients heterozygous state [29] and the difference between *in vivo* and *in vitro* secretion [30]. Moreover, the increased clearance of patients II-V plasma VWF also contributes to increase this discrepancy.

We have performed a first set of experiments to clarify the role of these two mutations by evaluating the capacity of WT and mutant (A1377V, R1379C and A1377V-R1379C) rVWF to bind rGpIbα. At 0.25 mg/mL of ristocetin, the R1379C rVWF bound rGpIbα with higher affinity than the WT (12-fold vs. 6-fold increased, respectively), whereas A1377V rVWF showed a 7.5-fold increased binding. Therefore, p.Ala1377Val does not compromise the VWF capacity to bind GpIbα, in agreement with the reported patients' biochemical data [27]. As expected, A1377V-R1379C rVWF showed a strongly reduced binding to rGpIbα, demonstrating a synergistic effect of p.Ala1377Val and p.Arg1379Cys that resulted in a 2M phenotype.

We subsequently evaluated, the hybrid A1377V-R1379C/WT rVWF, that unexpectedly showed only a slightly reduced binding to rGpIbα in comparison to WT. However, we observed similar results by testing patients' plasma VWF vs. a normal pooled plasma. This led us to suppose that the static conditions of the assay might allow the patients' plasma VWF to bind rGpIbα, although patients' VWF:RCo were markedly reduced.

The platelet-dependent VWF activity of patients' plasma and rVWF was evaluated using the VWF:GPIbM assay. The VWF:GPIbM values were similar to VWF:RCo for patients I, III, IV and V, whereas it was slightly higher for patient II (probably due to the type 2B p.Pro1266Leu mutation [22]). Also in this case, the A1377V-R1379C/WT rVWF had a binding capacity similar to the WT (0.86 vs. 1 VWF:GPIbM/VWF:Ag ratio). Therefore, the results obtained by testing the A1377V-R1379C/WT are probably due to a higher proportion of the WT subunit within this hybrid rVWF. Nevertheless, the binding of the A1377V-R1379C rVWF, to both WT and gain-of-function mutant rGpIbα proteins is strongly compromised.

In conclusion, these results demonstrate that the patients' 2M phenotype is due to the presence of both mutations (synergistic effect) and not to the p.Ala1377Val alone or to an hypothetical unidentified third mutation not located in the A1 domain.

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SUPPLEMENTARY DATA

Appendix S1. Phenotype and genotype data of available patient's family members.

Family member	ABO	BSS	FVIII:C *(IU/dL)*	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:CB *(IU/dL)*	VWF:RCo/ VWF:Ag	VWF:CB/ VWF:Ag	VWFpp/ VWF:Ag
Pt II Mother	O	6	75	42	11	28	0.26	0.7	2.4
Pt IV Father	n.d.	5	42	29	11	20	0.38	0.7	3.1
Pt V Son	n.d.	10	33	14	<6 *	11	0.21	0.8	n.d
NR	-	-1 to 3	50-150	40-169 [A] 55-165 [B]	41-160 [A] 53-168 [B]	45-170 [A] 56-174[B]	> 0.6	> 0.6	0.5-1.5

ABO, blood-group system; BSS, Bleeding Severity Score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:CB, von Willebrand factor collagen-binding activity; VWFpp, von Willebrand factor propeptide; NR, normal range; n.a., not applicable; n.d. not determined.

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Platelet VWF:Ag *(IU/10⁹ platelets)*	Platelet VWF:RCo *(IU/10⁹ platelets)*	Nucleotide Change	Amino Acid Change
0.19	0.14	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
n.d	n.d	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
n.d	n.d	n.d	n.d
0.22-0.60	0.15-0.58	n.a.	n.a.

* A VWF:RCo of 3 IU/dL was considered, in those cases with a VWF:RCo < 6 IU/dL, to perform the calculation of VWF:RCo/VWF:Ag ratio. Patients' plasma and platelets were tested only once. [A] Range values of normal individuals with blood group O; [B] range values of normal individuals with blood group non-O.