A novel p.C1130S mutation in a Finnish family with a complex phenotype of von Willebrand disease

Von Willebrand disease (VWD) is the most common inherited bleeding disorder and is caused by mutations that lead to either deficiency or dysfunction of von Willebrand factor (VWF). VWF is crucial for normal hemostasis as it mediates platelet adhesion and stabilizes coagulation factor VIII (FVIII). VWD patients typically suffer from mucocutaneous bleeds. Their disease is classified into type 1 or type 3, characterized by reduced or absent levels of VWF, respectively, or into type 2, which includes four subtypes and involves abnormal VWF function.[1,2] Although correct classification is important for optimal management of patients, allocation of individual patients into this classification system is not always straightforward due to overlapping clinical and/or laboratory phenotypes of the different (sub)types.[2] The multiple protein and glycan interactions of VWF underlie the heterogeneous molecular basis of VWD. In this letter, we present a family with a complex VWD laboratory phenotype and share our clinical and experimental insights of a novel VWF variant (i.e., p.C1130S), which was identified in all family members. All patients gave written informed consent, and this study was approved by the local ethical committee (HUS/1395/2018).

The family members (Figure 1) were recalled as part of a comprehensive re-evaluation of historical VWD diagnoses made in the Helsinki University Hospital Comprehensive Cancer Center.[3] The index patient, a 52-year-old male, was initially diagnosed with type 2A VWD after experiencing prolonged bleeding following an adenoidectomy. He was treated on-demand, first with cryoprecipitates and later with plasma-derived VWF:FVIII or desmopressin (DDAVP) with good efficacy. Currently, he reports only minor bleeding episodes, which are managed using tranexamic acid. His four children (three daughters and one son) were also diagnosed with VWD during their early childhood due to mucocutaneous bleeds and were treated on-demand with either plasma-derived VWF:FVIII, DDAVP and/or tranexamic acid.

We performed the following laboratory tests during the re-evaluation: platelet function analysis (PFA), FVIII activity (FVIII:C), VWF antigen (VWF:Ag), VWF platelet binding (VWF:GPIbM), VWF collagen binding (VWF:CB), FVIII binding of VWF (VWF:FVIIIB), VWF multimers and genetic sequencing of exons 2-52 of the *VWF* gene (Table 1). Briefly, the index patient showed a low VWF:Ag with a normal multimer pattern (*Online Supplementary Figure S1*), and a normal VWF:Ag/GPIbM ratio, suggesting a type 1 VWD. However, the severely reduced FVIII:C and VWF:FVIIIB laboratory phenotype resembled VWD type 2N rather than type 1. Additionally, VWF:CB and VWF:CB/Ag ratio were low. Similar observations were noted in all four children, but with higher FVIII:C and borderline-to-normal VWF:FVIIIB, resulting in a high VWF:FVIIIB/Ag ratio. Based on the results, the phenotype of the children most likely resembled a collagen-binding-dominant type 2M VWD, particularly given that both VWF:GPIbM/Ag ratio and multimer pattern were normal in combination with a low VWF:CB/Ag ratio.

Genetic analysis of the index patient revealed compound heterozygous variants in the *VWF* gene. The first variant was c.2561G>A (p.R854Q), located in exon 20 (VWF D'-domain), and is observed in up to 73% of all VWD type 2N patients. The change from an arginine to a glutamine at position 2,561 results in a loss of positive charge, which is thought to be responsible for the loss of FVIII binding to VWF.[4] The second variant was c.3388T>A (p.C1130S), located in exon 26 (VWF D3-domain), and represents a novel mutation. Interestingly, all four children were heterozygous for the novel c.3388T>A (p.C1130S) variant, while none of them carried the c.2561G>A (p.R854Q) variant.

Since all patients had low VWF:Ag, we evaluated *in vitro* VWF synthesis and secretion of both identified variants via transient transfection experiments in cultured Chinese hamster ovary K1 cells. Site-directed mutagenesis was used to introduce each of the two variants into full-length human wild-type (WT) VWF, using the pNUT-VWF-WT expression vector.[5] The primers used for each variant are given in *Online Supplementary Table S1*. The resulting plasmids pNUT-VWFR854Q and pNUT-VWFC1130S were used in transfection experiments. To mimic the heterozygous conditions in this family, we performed co-transfection experiments using a combination of pNUT-VWFR854Q and pNUT-VWFC1130S (the index patient) and a combination

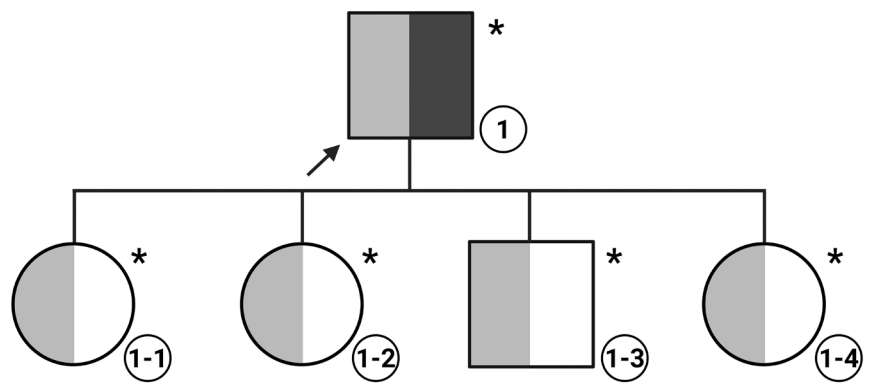

Figure 1. Family pedigree of the index patient. Males and females are indicated by squares and circles, respectively. The arrow points at the index patient of this report. All individuals were symptomatic and are indicated with an asterisk plus an identity label (1, 1-1, 1-2 ,1-3 and 1-4). Individuals carrying the c.3388T>A and c.2561G>A variant are represented by filled symbols in light gray and black, respectively. While the index patient carried both mutations on two different alleles, his four children carried the c.3388T>A variant on one allele in combination with a wild-type allele (white half). This figure was created using Biorender, the online scientific image and illustration software (*www.biorender.com*).

of pNUT-VWF-WT and pNUT-VWFC1130S (the children). For completeness, we also included co-transfections with pNUT-VWF-WT and pNUT-VWFR854Q and homozygous transfections with either pNUT-VWFR854Q or pNUT-VWFC1130S. As a negative control, mock transfections were performed without plasmids (mock control). Forty-eight hours after transfection, VWF:Ag levels in the collected medium were determined via an in-house enzyme-linked immunosorbent assay. Each transfection condition was performed five times and the mean VWF levels, corrected for transfection efficiency (*Online Supplementary Figure S2*), are depicted in Figure 2. Compared to WT *VWF* (set to 100%), homozygous expression of both the VWF p.R854Q and the *VWF* p.C1130S variant was significantly lower (45.7±9.3% and 35.7±8.6%, respectively, $P<0.0001$). Accordingly, co-transfection with p-NUT-VWFR854Q and p-NUT-VWFC1130S plasmids led to similar low VWF levels in the expression medium (45.9±13.2%). When the *VWF* p.R854Q or *VWF* p.C1130S variant was co-expressed with WT *VWF*, expression was partly recovered, resulting in VWF levels of 69.0±14.1% and 75.3±8.3%, respectively.

Remarkably, despite being heterozygous for the p.R854Q substitution, the index patient had undetectable VWF:FVIIIB

Table 1. Laboratory test results and genetic analysis of the index patient and his four children.

Laboratory test	Patient ID 1	Patient ID 1-1	Patient ID 1-2	Patient ID 1-3	Patient ID 1-4
Age at recall, years*	52	21	19	17	15
Sex	M	F	F	M	F
Blood group	A^-	A^+	A^+	O^+	A^+
PFA-200, sec C/EPI (nv: 82-150 sec) C/ADP (nv: 62-100 sec)	 >300 >300	 >300 >300	 >300 >300	 >300 >300	 >300 >300
VWF:Ag, IU/dL (nv: 50-190 IU/dL)	16	19	26	17	17
VWF:GPIbM, IU/dL** (nv: 50-190 IU/dL)	12	15	17	15	13
VWF:GPIbM/Ag ratio (nv: ≥0.7)	0.75	0.79	0.65	0.88	0.76
VWF:CB, IU/dL (nv: 50-172 IU/dL)	10	10	15	9	9
VWF:CB/Ag (nv: ≥0.7)	0.63	0.53	0.58	0.53	0.53
FVIII:C, IU/dL (nv: 52-148 IU/dL)	6	41	38	25	33
VWF:FVIIIB, IU/dL (nv: 50-100 IU/dL)	<10	63	67	49	51
VWF:FVIIIB/Ag ratio (nv: ≥0.75)	NC	3.32	2.58	2.88	3.00
Genetic analysis***	c.2561G>A c.3388T>A	c.3388T>A	c.3388T>A	c.3388T>A	c.3388T>A

*Recall in 2015. Fasting peripheral venous blood samples were collected via cubital venipuncture using a BD Vacutainer® system at a time remote from acute infection, inflammation, or surgery. Citrated whole blood samples were centrifuged at 2,000 g for 10 min and plasma aliquots were separated and stored at -70°C if testing could not be performed immediately. Platelet function was analyzed on a PFA-200® analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). Both VWF:Ag and VWF:GPIbM were measured on a BCS XP analyzer (Siemens, Marburg, Germany). A standard human plasma (ORKL 13, Siemens) was used as a reference for both assays. VWF:CB was assessed via a Technozym® kit (Technoclone, GmbH, Vienna, Austria) by an EVOLIS analyzer (Bio-Rad Laboratories, Berkely, CA, USA). VWF:FVIIIB was measured via the Asserachrom® VWF:FVIIIB assay (Stago Diagnostica, Düsseldorf, Germany). The binding of FVIII to VWF was compared to a normal control (set at 100 IU/dL), a homozygous type 2N control and a heterozygous type 2N control (Department of Clinical Chemistry, University and Regional Laboratories, Region Skåne, Sweden). FVIII:C was assessed using a one-stage assay and a Pathromtin SL and coagulation factor VIII deficient plasma (OTXW 13, Siemens) was used as a reference. **The VWF:GPIbM assay measures *VWF* binding to recombinant gain-of-function mutant GPIbα fragments. ***Genetic analysis included screening of exons 2-52 of the human *VWF* gene (East Genomic Laboratory Hub, Addenbrooke's Treatment Centre, Cambridge University Hospital NHS Foundation Trust, UK). Fluorescent sequencing analysis included Mutation Surveyor of exons 20 and 26 of the *VWF* gene. Reference values of each quantitative assay or ratio are given between round brackets. ID: identity; PFA: platelet function analyzer; C/EPI: collagen/epinephrine cartridge; nv: normal values; C/ADP: collagen/adenosine diphosphate cartridge; VWF: von Willebrand factor; Ag: antigen; GPIbM: platelet binding; CB: collagen binding; FVIII:C: factor VIII coagulant activity; FVIIIB: factor VIII binding, NC: not calculated.

levels. The recessive nature of VWD type 2N implies that the presence of either a homozygous 2N mutation or a compound heterozygous mutation with another mutation (i.e., type 2N or null allele) is necessary to cause a 2N phenotype. The p.C1130S substitution in the VWF D3-domain may also reduce FVIII binding to VWF, as this interaction relies on contact points within the D3-region.[6] The variant disrupts Cys[1130], which normally forms a disulfide bond with Cys[1126] or Cys[1149], potentially altering the domain's structure and impairing FVIII binding.[7] Along the same lines, substitution of Cys[1130] with a phenylalanine or arginine has been previously reported to result in impaired FVIII binding to VWF.[8,9] The loss of this cysteine could also explain the borderline VWF:FVIIIB values and lowered FVIII:C levels of the children in our study, due to their heterozygosity for the p.C1130S substitution.

One limitation of our study was the inability to reliably determine VWF:FVIIIB and assess VWF multimer distribution, owing to low VWF concentrations (i.e., low transfection efficacy) in the media. Besides the known effect of p.R854Q on FVIII binding, our data surprisingly suggest that this mutation may also impair *in vitro* VWF production or secretion. This finding aligns with other studies of reduced secretion of the p.R854Q variant after transient transfection in multiple cell lines.[10,11] Moreover, similar to our findings, homozygous expression of the recombinant variant p.R854W in HEK293 cells severely decreased the secretion of this variant into the medium, whereas co-transfection with WT *VWF* and p.R854W *VWF* yielded intermediate results.[11] In contrast, patients with type 2N VWD carrying the p.R854Q variant typically exhibit normal VWF:Ag levels, which may be due to heterozygosity permitting normal VWF production – except when there is a null allele – and the variant's specific impact on FVIII binding rather than on VWF production or secretion.

As mentioned, the novel p.C1130S variant, which was identified in all family members, leads to a loss in an intrachain disulfide bond, possibly locally distorting the protein's secondary structure.[7] Such misfolding may decrease the production or secretion, as observed in our experiments. Our finding of the novel variant impairing VWF production or secretion is corroborated by other studies showing intracellular retention of VWF variants in which the cysteines Cys[1130] or Cys[1149] are lost.[12,13] Although we and Tjernberg *et al.*[14] did not observe a dominant-negative effect of the loss of Cys[1130] on WT *VWF* when co-expressed, Eikenboom *et al.*[12] noticed that the p.C1149R variant inhibited the secretion of WT VWF subunits by as much as 35%. In all family members, we noticed much lower *ex vivo* VWF:Ag levels than we observed *in vitro* experiments. We hypothesize that this discordance may be due to increased *in vivo* clearance of the VWF variants. Indeed, a DDAVP trial in the index patient induced a strong initial response followed by a rapid decline in VWF:Ag levels 4 hours after the DDAVP (*data not shown*). Such a reduced *in vivo* survival of VWF after DDAVP administration has also been observed in patients carrying either the p.C1130F or p.C1149R variant.[8,15] Collectively, our findings support and extend existing evidence that the D3-domain constitutes a hotspot for VWF variants linked to increased clearance.

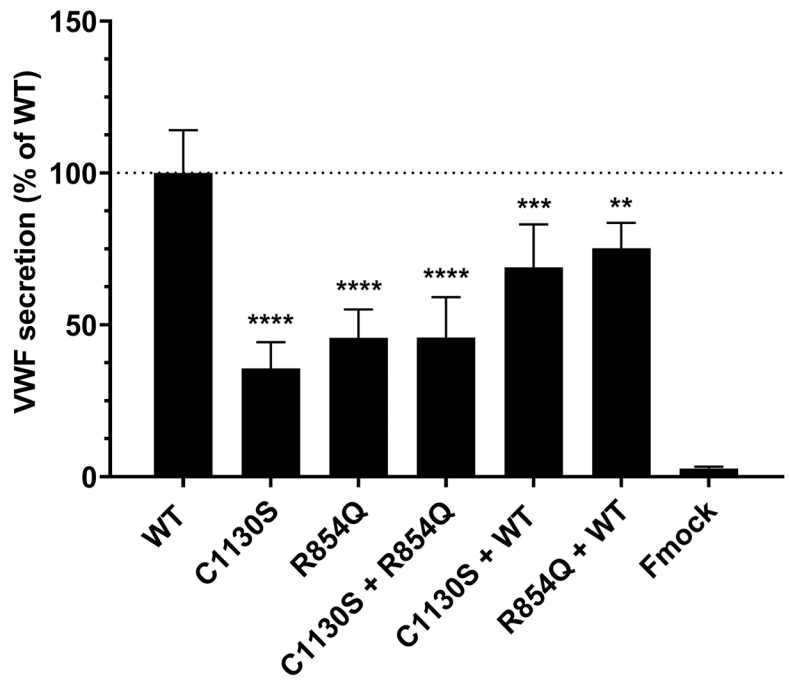

Figure 2. Von Willebrand factor secretion of transiently transfected Chinese hamster ovary K1 cells. Secretion levels of wild-type (WT) or variant von Willebrand factor (*VWF*) in the medium of transfected Chinese hamster ovary K1 cells were determined via enzyme-linked immunosorbent assay for VWF antigen (VWF:Ag) 48 hours after transfection. The VWF secretion levels were first corrected for the transfection efficacy of each condition before being expressed relative to the amount of secreted WT VWF. *VWF* secretion levels of an Fmock condition (pure jetPRIME® buffer) were measured as a negative control. The tested homozygous conditions included WT, C1130S and R854Q and heterozygous conditions included C1130S with R854Q, C1130S with WT and R854Q with WT. All experimental conditions were performed five times. Error bars represent mean ± standard deviation. Statistical analysis was performed using GraphPad Prism (v9.0.0 for Windows, GraphPad Software, USA) and compared to the WT condition; one-way analysis of variance: ****P<0.0001, ***P<0.001, **P<0.01 (P values <0.05 were considered statistically significant).

In summary, we highlighted the effects of a novel p.C1130S variant identified in a family with a complex VWD phenotype and showed that it causes reduced VWF production or secretion *in vitro*. Alone, or together with the p.R854Q variant, this novel variant most likely explains the low VWF:Ag and perhaps even the low VWF:FVIIIB levels observed in this family. As the D3-domain contributes to overall protein conformation, the p.C1130S variant may alter spatial arrangement of distal domains, possibly explaining the collagen-binding defect observed in the children. Future studies should focus on further unraveling the pathophysiological mechanisms by which both p.R854Q and p.C1130S variants affect VWF production, secretion and clearance by, for example, measuring intracellular VWF:Ag, analyzing VWF mRNA expression and/or pre- and post-DDAVP levels of VWF propeptide. The last is especially important since the results may have an impact on therapeutic guidance on DDAVP use in patients carrying the novel p.C1130S variant.

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Disclosures

No conflicts of interest to disclose.

Contributions

BC, IPo, TS, KV and SFDM conceived the study. BC, IPo, TS IPa, AV, A-EL, RL, KV and SFDM were responsible for the methodology. BC, IPo, TS, CT, KV and SFDM conducted the formal analysis. BC, IPo, TS, A-EL, RL and SFDM wrote the original version of the letter. BC, IPo, TS, IPa, AV, A-EL, RL, CT, KV and SFDM reviewed and edited it. BC, IPo, TS and SFDM were responsible for visualization. TS, CT, KV and SFDM supervised the work. TS and SFDM acquired funding. All authors read and agreed to this written manuscript.

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Data-sharing statement

Original data and protocols can be obtained upon request to the corresponding author.

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