Supplemental Data

Variable content of von Willebrand factor mutant monomer drives the phenotypic variability in a family with von Willebrand disease

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MATERIALS and METHODS

Patients. Individuals from this family affected with clinical bleeding had been diagnosed with von Willebrand disease (VWD). However, despite having the same mutation, different individuals in the family were given different diagnoses because of differences among them in biochemical parameters. Diagnoses included VWD type 1, type 2A and type 2M. Bleeding patterns also varied. Two individuals (II.1 and II.3) were given a desmopressin trial and their VWF levels increased by 20%, which was not clinically significant. Therefore, no desmopressin was offered to the rest of the family as a therapeutic option. All patients respond well to VWF concentrates and antifibrinolytic agents.

VWF antigen (VWF:Ag). VWF:Ag was measured on standard ELISA plates coated with a combination of two monoclonal antibodies as previously described. Briefly, plasma samples were plated into duplicate wells at three different dilutions per sample. Captured VWF was detected with a polyclonal rabbit antibody conjugated with horse-radish peroxidase. Agreement in measured concentrations at different dilutions was evaluated as a measure of quality.

Ristocetin cofactor activity (VWF:RCO). VWF:RCo was measured by automated analysis of formalin-fixed platelet agglutination on a Dade-Behring BCS analyzer (Dade Behring Inc.). Data were analyzed using the manufacturer’s end-point based software package.

VWF propeptide (VWFpp) level. VWFpp was captured using monoclonal antibodies, 239.2 and 239.3, and detected using a rabbit anti-VWFpp polyclonal antibody, Mango. Immune complexes were detected using biotin-conjugated goat anti–rabbit IgG (Pierce), avidin-horseradish peroxidase, and o-phenylenediamine substrate (Pierce).

Binding to recombinant GP Ibα. The assay was performed as previously described. Briefly a gain-of-function GPIbα construct (tGPIbα235Y;239V) was synthesized containing 2 mutations, D235Y and M239V. A monoclonal antibody against human GPIbα was bound to a 96-well
enzyme-linked immunosorbent assay (ELISA) plate (Immulon 4 HBX, Thermo Scientific) and incubated at 4°C overnight. The tGPlbα235Y;239V construct was then added and incubated at room temperature for 1 hour. Diluted plasma was added as the VWF source and also incubated at room temperature for 1 hour. A mixture of biotinylated monoclonal anti-VWF antibodies (AVW-1 and AVW-15) was used to detect the presence of VWF. Streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate was added and the optical density measured on an ELISA reader.

**Binding to collagen III and collagen VI.** Binding of von Willebrand factor to collagen was performed using recombinant VWF or patient plasma in an ELISA assay. Nunc Maxisorp plates (Nalge Nunc) coated with 1 μg mL⁻¹ human placental collagen (Southern Biotech, Birmingham) and Pierce Amine Binding Maleic Anhydride ELISA plate (Thermo Scientific) coated with human type VI collagen (Southern Biotech) were used in type III and type VI collagen binding assays, respectively. The bound VWF was detected with biotinylated VWF antibody (DAKO) in type III collagen binding assay or with a combination of AVW-1 and AVW-15 (biotinylated) for type VI collagen binding assay. Streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate (PNPP) were added and the optical density measured on an ELISA reader.

**Factor VIII coagulant activity (FVIII:C).** FVIII:C was determined by one-stage clotting assay in an MLA 900 analyzer using a "lupus-anticoagulant" insensitive activator reagent (Actin FS). Samples were run at three dilutions and results were compared to a standard curve. FVIII:C was reported in relation to a population reference range.

**VWF multimer analysis.** VWF multimer structure was analyzed in all affected individuals. The multimer pattern was evaluated by 1.5% agarose gel electrophoresis followed by Western blotting with a polyclonal VWF antibody, as described.⁴
ADAMTS13 activity. ADAMTS13 activity in plasma was determined using a peptide substrate derived from the VWF A2 domain as described previously. Pooled normal plasma was used as a standard.

AU/VWFα-11 nanobody binding. Assay was performed as previously described. Briefly, the llama nanobody AU/VWFα-11 was immobilized on an ELISA plate as a capture antibody, and pooled normal plasma or patient plasma in serial dilutions was then added to the coated wells. Bound VWF was detected by an HRP-conjugated polyclonal VWF antibody (DAKO). Captured VWF was plotted against the concentration of the VWF added to the wells, and the slopes of the plots from patient samples were divided by that of the pooled plasma and the ratios were shown.

Recombinant VWF expression in mammalian cells. The plasmids containing wild-type human full-length VWF cDNA, either with myc and His tags (WT-mycHis) or no tag (WT-pCIneo) were used in this study. The M1304R (T3911G) mutation was introduced into WT-pCIneo by site-directed mutagenesis to create M1304R-pCIneo. Human embryonic kidney cells (HEK293T, Dr. Ginsburg, University of Michigan, Ann Arbor, MI) or HEK293 cells (American Type Culture Collection® CRL-1573.3™) were cultured for transfection at 37°C in an atmosphere of 5% CO₂ as described. The M1304R-pCIneo was either transfected alone or co-transfected with WT-mycHis into either HEK293 or HEK293T cells. The transfection conditions were described in previous study. Conditioned media were harvested for the two-color multimer analysis 72 hours after transfection. The transfected cells were washed twice with phosphate-buffered saline (PBS) and lysed in Reporter Lysis Buffer (Promega). The concentration of VWF:Ag in conditioned media (supernatant) and cell lysates was determined using standard ELISA.

Confocal immunofluorescence microscopy. The intracellular localization of VWF was examined by immunofluorescent staining and the images were collected by confocal laser scanning microscopy using an Olympus FluoView FV1000 confocal laser-scanning
HEK293 cells (CRL 1573; American Type Culture Collection) were cultured at 37°C in a 5% CO₂ atmosphere in Minimal Essential Medium (Mediatech) supplemented with 10% FBS (Invitrogen). Cells were transfected with the use of TransIT-293 (Mirus), according to the manufacturer's instructions, and stained for immunofluorescence microscopy as described previously. Forty-eight hours after transfection, cells were fixed with a 3.7% buffered formalin solution, permeabilized with 0.5% Triton X-100 in 20 mM HEPES, 300 mM sucrose, 50 mM NaCl and 3 mM MgCl₂·6H₂O (pH 7.0), and blocked with 2% normal goat serum in phosphate-buffered saline (PBS). Cells were incubated overnight in primary antibody, polyclonal anti-VWF (Dako), at 3 μg mL⁻¹, and this was followed by washes with PBS. Cells then were incubated in secondary antibody, AlexaFluor-488-conjugated goat anti-rabbit IgG (H + L) [F(Ab′)2] (Invitrogen) diluted 1 : 1000, for 30 min, and this was followed by washes with PBS. Cells were placed on coverslips using Vectashield (Vector Laboratories).

**Two-color multimer analysis.** Recombinant VWF in the cell supernatants was analyzed by electrophoresis through a 2% high-gelling-temperature agarose-resolving gel (Lonza) containing 1% SDS and transferred to Immobilon-FL (Millipore) by electroblotting at 100V for 1 hour in 25 mM Tris, 200 mM glycine, 20% methanol, and 0.03% (wt/vol) SDS. Membranes were either blocked with Odyssey Blocking Buffer (LI-COR) then incubated with AVW-5 followed by incubation with IRDye 680–conjugated goat anti-mouse IgG, or blocked with Affinity BioReagents then incubated with rabbit-anti–c-Myc followed by incubation with IRDye 800–conjugated goat anti–rabbit IgG (LI-COR).

**Ristocetin- or botrocetin-induced platelet binding to recombinant VWF.** Recombinant VWF protein (60 mU/mL) is incubated with VWF antibody (6 μg/mL, DAKO) that conjugated with AlexaFluor 647 (Life Technologies). Formalin-fixed platelets were added to the VWF/antibody mixture before the samples were incubated with different concentrations of botrocetin or ristocetin for 30 – 60 min at room temperature. The samples were diluted with 300 uL PBS and analyzed on the LSRII flow cytometer (BD Biosciences). Formalin-fixed platelets were
resuspended in 2% BSA in PBS at a concentration of 2 x 10^5/μL and 1 x 10^6 platelets were used in each samples.

**Allele-specific primer extension.** Blood samples were collected and stored in RNA later (Ambion, Austin, TX) per manufacturer recommendations. Total cellular RNA was isolated using a Ribopure Isolation kit (Ambion). cDNA was synthesized from 200 ng of total RNA using iScript cDNA kit (BioRad). Standard locus-specific PCR using exon 28 primers (5'-GAGCCACCCTCTGTATG-3' and 5'-TGCCCCCATCTTCACCTC-3') was performed. Allele-specific primer extension was performed with 0.15 μM of 6-FAM-labeled probe (5'-TGAAGTGCTGAAGGCCTTTGTGGTGGACAT-3'), mixing with Qiagen PCR buffer at 1.5 mM MgCl₂ (Qiagen), 200 μM nucleotide mixture of dATP, dGTP, dCTP and ddTTP (GE Amersham), 2.5U Taq polymerase (Epicentre Biotechnologies), and 2 μl of ExoSap-treated PCR product and amplified via an initial denaturation at 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and a final extension of 72°C for 7 minutes. The resulting extension product(s) are differentially sized (33bp or 42bp) because of the 3911T>G mutation. Products were diluted 1:10 in Hi-Di formamide, separated by capillary electrophoresis on an Applied Biosystems 3100-Avant Genetic Analyzer, and analyzed with GeneMapper 3.5 (Applied Biosystems). Relative allele-specific mRNA accumulation was determined using the primer extension protocol with the fluorescently labeled probe and minigene plasmid constructs (Integrated DNA Technologies) containing each homozygous allele (T or G) of the 3911 T>G SNP. Each clone was quantified with PicoGreen (Invitrogen) and mixed in specific ratios to generate a 10-point standard curve. All separations were completed in quadruplicate. The standards were fit to a line resulting in an R² value of 0.996, calculated by linear regression with Microsoft Excel.

**Unperturbed molecular dynamics (MD) simulations.** The MD simulations were performed with the program NAMD\(^\text{10}\) using the CHARMM all-hydrogen force field (PARAM22)\(^\text{11}\) and the TIP3P model of water. The protein was solvated in a cubic water box with side length of 84 Å.
Chloride and sodium ions at concentrations of 150 mM were added to neutralize the charges of the protein. To avoid finite size effects, periodic boundary conditions were applied. After solvation, the system underwent 500 steps of minimization while the coordinates of the heavy atoms of the protein were held fixed and subsequent 500 steps with no restraints. Electrostatic interactions were calculated within a cutoff of 10 Å, while long-range electrostatic effects were taken into account by the Particle Mesh Ewald summation method. Van der Waals interactions were smoothly turned off using a switch function starting at 8 Å and ending at 10 Å. The dynamics were integrated with a time step of 2 fs using SHAKE to rigidly constrain hydrogen atoms. Snapshots were saved every 10 ps for trajectory analysis. Before production runs, harmonic constraints were applied to the positions of all heavy atoms of the protein to equilibrate the system at 300 K during a time length of 0.2 ns. For the simulation with the mutant, harmonic constraints were kept on all heavy atoms except those of the mutated residue and of amino acids where any heavy atom is at least 4 Å from any heavy atom of the mutated residue, and equilibration was continued for another 2 ns. After this equilibration phase, the harmonic constraints were released. One simulation was performed with the wild-type and one with the M1304R mutant. Each run lasted 50 ns. During all runs, the temperature was kept constant at 300 K using the Langevin thermostat with a damping coefficient of 1 ps⁻¹, while the pressure was held constant at 1 atm by applying a pressure piston.

**Free energy perturbation calculations.** Using molecular dynamics (MD) simulation to predict folding energy (ΔGfold) of the mutant A1 domain is challenging because an exhaustive sampling of the free energy landscape of the protein is computationally prohibitive. In this study, we combine the thermodynamic cycle (Supplemental Figure S1) and free energy perturbation (FEP) simulations to estimate of the difference in folding energy (ΔΔG) between the mutant and wild-type A1 domains. Dual-topology approach was used in the free energy perturbation calculations, where the initial and final states are defined. In this case, the initial state is Met1304 and the final state is Arg1304 or Val1304, respectively. A MD simulation is performed
to slowly change atoms belonging to Met1304 to the atoms of Arg1304 or Val1304, respectively [for example, from (\(-\text{Met-Met-Glu}\)\)\text{folded} to (\(-\text{Met-Arg-Glu}\)\)\text{folded} in Supplemental Figure S1)]. The free energy difference between the two states (\(\Delta G_{\text{alch}}^2\) in Supplemental Figure S1) was calculated using alchemical transformation.\(^{18}\) An illustration of the method can be found here: http://www.ks.uiuc.edu/Research/namd/2.9/ug/node58.html. Each FEP run lasted 1 ns and contained 40 intermediate states. Each intermediate state consisted of 12.5 ps of equilibration and 12.5 ps of data collection. A soft-core term\(^ {19}\) was used to avoid singularities in the van der Waals potential. In total, three FEP runs were performed with the folded state of the protein. They were started from snapshots collected after 10, 20 and 30 ns, respectively, during the 50-ns simulation with the wild-type A1.

In order to perform FEP in the unfolded state, the assumption was made that when the protein is denatured the mutation site is completely solvent exposed and can be approximated by a tripeptide consisting of the mutated side-chain and the neighboring residues. The resulting tripeptide Met1303-Met1304-Glu1305 was solvated in a cubic water box of 40 Å side length and one FEP run was performed. In order to improve the accuracy of the calculated free energy change, each FEP simulation was performed in both forward and backward directions, i.e., residue 1304 was mutated from the wild-type to the target residue and back to the wild-type. A weighted average was then calculated for all values sampled from the forward and backward simulation in each collection window using the Bennett acceptance ratio method,\(^ {20}\) which has been shown to yield the most precise free energy values.\(^ {21}\) The total free energy change in a specific simulation was then calculated as the sum of the free energy changes in each collection window. Once the free energy changes were calculated for the folded and unfolded state, the thermodynamic cycle (illustrated in Supplemental Figure S1) was used to calculate \(\Delta \Delta G\) as follows:

\[
\Delta \Delta G \text{ from FEP calculation:} \\
\Delta \Delta G = \Delta G_{\text{fold}}^2 - \Delta G_{\text{fold}}^1
\]
\[ \Delta G_{\text{alch}}^2 \cdot \Delta G_{\text{alch}}^1 \]

Unfolded state (Met->Arg in tri-peptide, solvent exposed): \( \Delta G_{\text{alch}}^1 = -261.55 \) kcal/mol

Folded state (Met->Arg in protein): \( \Delta G_{\text{alch}}^2 \) in three runs:

<table>
<thead>
<tr>
<th>Run</th>
<th>( \Delta G_{\text{alch}}^2 )</th>
<th>( \Delta G_{\text{alch}}^1 )</th>
<th>( \Delta \Delta G )</th>
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<tr>
<td>Run 1</td>
<td>-219.87</td>
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</tr>
<tr>
<td>Run 2</td>
<td>-229.67</td>
<td>-261.55</td>
<td>31.88</td>
</tr>
<tr>
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<td>-228.59</td>
<td>-261.55</td>
<td>32.96</td>
</tr>
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<td></td>
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</tr>
<tr>
<td>SEM:</td>
<td></td>
<td></td>
<td>3.10</td>
</tr>
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</table>

Unfolded state (Met->Val in tri-peptide, solvent exposed): \( \Delta G_{\text{alch}}^1 = 2.7 \) kcal/mol

Folded state (Met->Val in protein): \( \Delta G_{\text{alch}}^2 \) in three runs:

<table>
<thead>
<tr>
<th>Run</th>
<th>( \Delta G_{\text{alch}}^2 )</th>
<th>( \Delta G_{\text{alch}}^1 )</th>
<th>( \Delta \Delta G )</th>
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<tr>
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<td>7.98</td>
<td>2.7</td>
<td>5.28</td>
</tr>
<tr>
<td>Run 3</td>
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<td>2.7</td>
<td>4.17</td>
</tr>
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<tr>
<td>SEM:</td>
<td></td>
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</table>

Supplemental Figure S1. Thermodynamic cycle of the folding reaction of the A1 domain.

The thermodynamic cycle allows estimation of the folding \( \Delta \Delta G \) in the following way:

\[ \Delta \Delta G = \Delta G_{\text{fold}}^2 - \Delta G_{\text{fold}}^1 = \Delta G_{\text{alch}}^2 - \Delta G_{\text{alch}}^1 \]
$\Delta G_{\text{fold}}^1$ is calculated from the FEP run using the tri-peptide containing the mutated side chain, which mimics the unfolded state by completely exposing the mutation site to solvent. $\Delta G_{\text{fold}}^2$ is calculated from the FEP runs of the folded state.

**Bleeding Scores.** We used 2 different bleeding tools to assess clinical bleeding. The International Society of Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) which is referenced in the main text (reference #16) and the CDC based questionnaire. This questionnaire consists of 12 questions that were selected from a more extensive set of questions on the basis of their positive predictive value focusing on nosebleeds, minor injury (cuts), bruising, gingival bleeding, bleeding after dental and surgical procedures, and transfusion requirements. Questionnaires were graded on a Likert scale with the lowest (best) possible score being 0 and the highest (worst) score being 7. To evaluate sensitivity and specificity, the questionnaire was also given to a group of unrelated patients with VWD recruited from the Bleeding Disorders Clinic at the University of Iowa and a group of unrelated controls. A full copy of the questionnaire is as follows.
QUESTIONNAIRE from CDC—BLEEDING HISTORY

NOSEBLEEDS

1. How many nosebleeds would you say you have had in your entire life? Would you say…
   - More than 20
   - 10-19
   - 5-9
   - None
   - Do not know
   - Refuse to answer

IF NO NOSEBLEEDS SKIP TO 3

2. Do you experience more nosebleeds, fewer nosebleeds, or about the same number of nosebleeds when you are using a lot of aspirin?
   - More
   - Same
   - Fewer
   - Do not know
   - Refuse to answer

BLEEDING AFTER MINOR INJURY:

3. Compared to most people, when you get a minor cut, do you think that it takes longer for the bleeding to stop, about the same amount of time for the bleeding to stop, or not as long for the bleeding to stop?
   - Longer
   - About the Same
   - Not as long
   - Do not know
   - Refuse to answer

4. Compared to most people, when you get a minor cut, do you think that you bleed more heavily, about the same, or less heavily? (Please consider the amount of bleeding, not the length of time you bleed).
   - More Heavily
   - About the Same
   - Less Heavily
   - Do not know
   - Refuse to answer

5. Compared to most people, when you bite your lip, do you think you bleed more, about the same, or less? (Please consider both the amount of bleeding and the length of time you bleed).
   - More
   - About the Same
   - Less
   - Do not know
   - Refuse to answer
6. Compared to most people, when you bite your tongue, do you think you bleed more, about the same, or less? (Please consider both the amount of bleeding and the length of time you bleed).
   - More
   - About the Same
   - Less
   - Do not know
   - Refuse to answer

BRUISING
7. How often do you notice bruises in places that you can’t recall having hurt yourself? Would you say...
   - Everyday
   - Most days
   - At least once a week
   - At least once a month
   - Several times a year
   - Less than once a year
   - Do not know
   - Refuse to answer

EXCESSIVE GUM BLEEDING
8. When you go to the dentist, about how much do you bleed from your gums? Would you say it is more than most people your age, about the same as most people your age, or less than most people your age?
   - More than most people
   - Same as most people
   - Less than most people
   - Do not know
   - Refuse to answer

9. When you brush your teeth, about how often do you notice bleeding from your gums? Would you say...
   - Everyday
   - Most days
   - At least once a week
   - At least once a month
   - Several times a year
   - Less than once a year
   - Do not know
   - Refuse to answer

BLEEDING AFTER DENTAL PROCEDURES:
10. Have you ever had a tooth extracted or dental surgery?
    - Yes (go to 10a)
    - No (go to 11)
    - Do not know (go to 11)
    - Refuse to answer (go to 11)

10a How many times have you had a tooth extracted or dental surgery?
    - Times: 
    - Do not know
    - Refuse to answer
10b. Did you ever have problems with bleeding following a tooth extraction or dental surgery? (Did the doctor or you have to do something to stop the bleeding?)
   - Yes
   - No
   - Do not know
   - Refuse to answer

10c. How many times did you have problems following dental surgery?
   - Times: __________
   - Do not know
   - Refuse to answer

BLEEDING AFTER SURGICAL PROCEDURES:

11. Have you ever had surgery or an operation other than dental surgery?
   - Yes (go to 11a)
   - No (go to 12)
   - Do not know (go to 12)
   - Refuse to answer (go to 12)

11a. How many times have you had surgery?
   - Times: __________
   - Do not know
   - Refuse to answer

11b. Did you ever have problems with bleeding when you had surgery? (Did the doctor or you have to do something to stop the bleeding?)
   - Yes (go to 11c)
   - No (go to 12)
   - Do not know (go to 12)
   - Refuse to answer (go to 12)

11c. How many times did you have a problem with bleeding following surgery?
   - Times: __________
   - Do not know
   - Refuse to answer

MEDICAL TREATMENT TO STOP BLEEDING

12. Now, I am going to ask you about other times when you may have required medical treatment to stop bleeding. Please do not include any treatments you may have had after surgery or dental surgery. Have you ever required medical treatment to stop bleeding?  This includes times you may have injured yourself, received vaccinations or injections, or had any part of your body pierced.
   - Yes
   - No
   - Do not know
   - Refuse to answer
### Supplemental Table S1: Demographic parameters and VWF measurements of the studied family

<table>
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<th>Individual</th>
<th>M1304R</th>
<th>Age</th>
<th>Sex</th>
<th>BS-CDC</th>
<th>ISTH-BAT</th>
<th>Blood type</th>
<th>VWF:Ag</th>
<th>RCo</th>
<th>VWF/Ag</th>
<th>FVIII</th>
<th>VWFpp</th>
<th>CB&lt;sub&gt;IIa&lt;/sub&gt;/ VWF:Ag</th>
<th>CB&lt;sub&gt;IIa&lt;/sub&gt; binding</th>
<th>CB&lt;sub&gt;IIIa&lt;/sub&gt;/ VWF:Ag</th>
<th>CB&lt;sub&gt;IIIa&lt;/sub&gt; binding</th>
<th>GPIb&lt;sub&gt;α&lt;/sub&gt; binding</th>
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<td>63</td>
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<td>7</td>
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**BS-CDC:** bleeding scores based on the questionnaires from CDC; **ISTH-BAT:** bleeding scores based on the International Society of Thrombosis and Haemostasis Bleeding Assessment Tool; **VWF:Ag:** VWF antigen (IU/dL), normal range (NR): 50 – 240 IU/dL; **RCO:** ristocetin cofactor (IU/dL), NR: 54 – 279 IU/dL; **FVIII:C:** factor VIII coagulant activity (IU/dL), NR: 63 – 205 IU/dL; **VWFpp:** VWF propeptide level (IU/dL), NR: 62 – 183 IU/dL; **CB<sub>IIa</sub> binding:** binding to collagen type III (IU/dL), NR: 59 – 249 IU/dL; **CB<sub>IIIa</sub> binding:** binding to collagen type VI (IU/dL); **GPIb<sub>α</sub> binding:** binding to recombinant GPIb<sub>α</sub> (IU/dL); **ADAMTS13 activity:** expressed as a percentage of the ADAMTS13 activity in pooled normal plasma, which is 100%. **NA:** data are not available.
### Supplemental Table S2: Genotypes of selected VWF SNP's in affected members of the family

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*Base Pair change
‡ Amino acid Change
† Reference SNP number
Supplemental Figure S2: Allele-specific VWF mRNA levels. Allele-specific mRNA levels are represented as percent T allele (normal allele at base 3911) along the y-axis. Individual pedigree members are represented along the x-axis. All samples were quantitated in quadruplicate, and each bar represents the mean percentage of the normal allele with standard deviation. VWF mRNAs of unaffected relatives are clustering around 95% of normal allele and those of VWD patients around 76%. Note that the percentage of normal allele in unaffected individuals does not reach 100% due to background noise in the assay.
Supplemental Figure S3. Nanobody binding of recombinant VWF. **Upper panel:** nanobody binding. DNAs of either wild-type or mutant VWF were either transfected alone or together into HEK293T cells and binding of recombinant VWF in the cell-cultured medium to AU/VWFa-11 nanobody was examined. **Lower panel:** VWF multimers. The binding of nanobody is higher for the mutant VWF than the wild-type (lane 1 vs lane 5 in the lower panel), even though the multimer size of mutant VWF is smaller than the wild-type.
Supplemental Figure S4. VWF multimers in plasma of some of the affected members from a different blood draw. VWF multimers were analyzed by electrophoresis on agarose SDS gels and detected by a polyclonal antibody to human VWF. The affected individuals with similar VWF multimer patterns to those shown in Figure 2B are 1) III.3 with multimers similar to normal; 2) II.3, II.5, III.4, and III.2 without high-molecular-weight multimers. Those with different multimer patterns from Figure 2B are 1) II.1 and III.6, changed from missing the high-molecular-weight multimers to normal multimers; 2) III.7, changed from normal multimers to missing high-molecular-weight multimers.
Supplemental Figure S5. High resolution of VWF multimers of the affected individuals.

**Upper panel:** Densitometry quantification of satellite bands. **Lower panel:** VWF multimers on a high-resolution agarose gel. VWF in plasma from normal and affected individuals was analyzed by electrophoresis on a 2.5% agarose SDS gel and detected by a polyclonal antibody to human VWF. Plasma from individual II.7 was no longer available.
Supplemental Figure S6: Schematic representation of the biosynthetic defects caused by a mutation in VWF. The biosynthetic defects of a mutant VWF may be the consequence of one or more of the following: A. The mutant allele is hypomorphic resulting in lower steady-state mRNA levels due to decreased transcription or increased mRNA degradation. This leads to fewer VWF mutant monomers being synthesized; B. Degradation of VWF mutant monomers and dimers may be different among affected individuals. The normal monomers are depicted in blue and the mutant monomers in cyan. The VWF mutant monomers may be less stable than
the normal resulting in increasing degradation of the mutant monomers by protein quality control system in the endoplasmic reticulum (ER). Dimerization occurs between normal monomers, normal and mutant monomers, and mutant monomers. C. Incorporation of mutant-containing dimers into VWF multimers may vary among different individuals.
REFERENCES


