A Novel Type 2A von Willebrand Factor Mutation Located at the Last Nucleotide of Exon 26 (3538G>A) Causes Skipping of Two Non-Adjacent Exons

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Abstract

In this manuscript, we describe a case of Type 2A VWD caused by the novel heterozygous G→A transition at nucleotide 3538, which should result in the putative, non-conservative substitution of G1180R. This mutation was reproduced by site-directed mutagenesis; however, the recombinant mutant protein was efficiently secreted from cells and assembled correctly into multimers. Because the substitution is located at the last nucleotide of exon 26, the patient’s platelet VWF mRNA was analyzed and three transcripts were observed: the normal transcript without the 3538G>A transition, a transcript with the inframe deletion of exon 26 and a transcript with the inframe deletions of exons 23 and 26. These deletion VWF cDNA constructs were created and the resulting recombinant proteins were analyzed following transfection into COS-7 cells. Co-transfection results demonstrate that the exon skipped transcripts lead to intracellular retention and the levels of VWF:Ag produced by these constructs were as follows: del23/26 < del26 < G1180R ≤ wild type. The homozygous exon skipped transcripts show the presence of only the lowest molecular weight multimers. The G>A transition at nt 3538 does not result in the expression of the G1180R missense mutation, but rather leads to exon skipping which is the pathogenic basis of the patient’s phenotype. This is the first report of a coding region mutation resulting in the skipping of 2 non-adjacent exons.
Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder known in humans, with published prevalence rates between 0.01% and 1%.\(^1,\(^2\) The clinical spectrum of the disease is broad, ranging from mild mucocutaneous bleeding observed in Type 1 VWD (quantitative deficiency of von Willebrand factor - VWF) to severe, spontaneous bleeding in Type 3 VWD (complete absence of VWF). Type 2 VWD is characterized by the presence of qualitatively abnormal VWF and is a heterogeneous condition both phenotypically and genotypically. Four distinct subtypes are recognized.\(^3\) Type 2A and Type 2B VWD are characterized by the loss of high molecular weight VWF multimers; however, these subtypes are best distinguished based on VWF interactions with platelets; this interaction is increased in Type 2B VWD as is observed by the enhanced ristocetin-induced platelet agglutination, and significantly reduced in Type 2A disease. Type 2M VWD refers to qualitatively abnormal variants with decreased platelet-dependent function not associated with the loss of high-molecular weight multimers. Type 2N VWD demonstrates defective binding of VWF to factor VIII.

The accurate discrimination of subtypes is important, particularly when it affects patient management. This has become easier since our understanding of the condition has progressed. However, challenging cases remain, particularly when the diagnosis is based on phenotypic data alone. Coagulation based laboratory tests vary over time within individuals, and an accurate diagnosis is sometimes difficult to achieve, even when repeated tests are performed. The
VWF gene was cloned in 1985\(^4-7\) enabling the identification of the molecular genetic basis in some of the subtypes. The ability to combine phenotypic and genotypic information has helped to improve the accuracy of diagnosis.

The inheritance pattern of Type 2A VWD is usually autosomal dominant and significant progress has been made in defining the molecular genetic pathology of this subtype. Missense mutations account for the majority of Type 2A cases, and most are located within the region of exon 28 that encodes the A2 domain. Type 2A mutations have also been described at the C-terminus of the protein and in the D2 and A1 domains. Two distinct pathogenic sub-groups have been described within Type 2A VWD\(^8, 9\); Group 1 is caused by defects in the synthesis and intracellular transport of multimers, and Group 2 results from an increase in plasma proteolysis of VWF by the ADAMTS13 protease following secretion into blood. Type 2A mutations can result in defective dimerization or multimerization and splicing defects found in the D3 domain leading to Type 2A have previously been described in abstract form.\(^10, 11\) VWF undergoes extensive post-translational modifications by initially dimerizing through multiple intermolecular disulfide bonds between the carboxyl-terminal ends of the protein and then, once transported to the Golgi, forming interdimer disulfide bonds using cysteines found in the D3 domain. The resulting multimers range in size from 500 – 20,000 kDa and it is the highest-molecular weight multimers that are most functionally active in terms of VWF's adhesive properties.

VWF is the largest soluble plasma protein. The VWF gene encompasses 178 kb of genomic DNA, and is comprised of 52 exons. Based on the number of
exons that have to be spliced together, it is not surprising that several splicing mutations resulting in various types of VWD have previously been described (http://www.shef.ac.uk/vwf/). Splicing defects are most often the result of a mutation in the 5’ (donor) or 3’ (acceptor) splice sites located at the exon/intron boundary, however, changes in the last nucleotide of an exon have also been shown to cause exon skipping in a number of conditions. In this study we describe a patient with a novel VWF mutation, 3538 G>A, which results in the in-frame exon skipping of exon 26, and exons 23 and 26. To our knowledge, this is the first description of skipping of two non-adjacent exons as a pathogenic mechanism in VWD or any other condition.

**Patients, materials and methods**

**Patients**

The study family was submitted to the Canadian Type 1 VWD Study as a potential Type 1 VWD family. Each individual was informed of the experimental nature of the study and gave their informed consent. The study was approved by the Institutional Review Board at Queen’s University, and at the source institution. To be eligible for the study at least one member from the family had to have a personal history of excessive mucocutaneous bleeding, von Willebrand factor antigen (VWF:Ag) and ristocetin cofactor activity (VWF:RCo) between 0.05 and 0.50 U/mL measured on at least 2 different samples. Whole blood samples for DNA extraction were collected in 3.2% sodium citrate (at a ratio of 9:1 vol/vol) from the index case and available immediate family members, and a plasma
sample was collected from the index case for repeat VWD phenotypic studies to be performed in a central laboratory.

**Coagulation studies**

Laboratory tests for VWF:Ag, VWF:RCo and factor VIII coagulant activity (FVIII:C) were performed at the source clinic attended by the patient, and were repeated on different plasma samples at the Clinical Hemostasis Laboratory at Kingston General Hospital (Canada). All available laboratory results were averaged. In the source clinic laboratories, VWF:Ag and VWF:RCo values were assayed using the locally validated methodology. In the central laboratory, VWF:Ag was measured using the IMMUBIND VWF enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (American Diagnostica, Greenwich, CT). The VWF:RCo was measured by platelet aggregometry using freshly prepared, washed normal platelets, and the FVIII:C was measured using a one-stage assay. All measurements were made against a non-ABO-matched commercial reference plasma that was calibrated against the 91-666 or 97-586 World Health Organization (WHO) Plasma Standard. Ristocetin induced platelet aggregation (RIPA) was performed on a BIO/DATA Platelet Aggregometer, model PAP-4. VWF multimers were analyzed by electrophoresis using a 1.6% sodium dodecyl sulfate (SDS) agarose gel followed by electrotransfer to a nylon membrane. The multimers were visualized using the chemiluminescent visualization kit from Amersham Pharmacia Biotech (Baie D'Urfe, PQ, Canada).
PCR amplification of genomic DNA and mutation identification

A blood sample was collected from the patient and each family member, and genomic DNA was isolated using a salt extraction method. DNA corresponding to the coding sequence of exons 18 – 52 of the VWF gene (encoding the mature VWF subunit) and at least 20 nucleotides of the adjacent intron were amplified by polymerase chain reaction (PCR). Primer sequences have been previously published. Additionally, genomic DNA was PCR amplified for introns 23, 25 and 26 and 300bp of the 3’ end of intron 22 (primer sequences Table 1). Using a DNA thermal cycler (Perkin Elmer Life Sciences, Shelton, CT), DNA was amplified for 35 cycles of 45 to 60 seconds at 94°C, 45 to 60 seconds at 53°C to 60°C, and 45 to 60 seconds at 72°C, and the amplified products were sequenced directly on an ABI model 373 automated sequencer (Cortec DNA Service Laboratories, Kingston, Ontario, Canada). All DNA sequences were compared with normal VWF DNA sequences with the assistance of Vector NTI Suite software (InforMAX, Inc, Bethesda, MD).

Platelet RNA isolation and amplification

A blood sample was collected in EDTA from the index case for VWF mRNA analysis. Platelet rich plasma was separated by centrifugation at 800 rpm for 5 minutes at room temperature. Platelets were then pelleted by centrifugation at 1400 rpm for 10 minutes at room temperature. The platelet pellet was washed twice with phosphate buffered saline (PBS) and the total RNA was extracted
using the QIAamp RNA Blood Mini Kit (QIAGEN, Mississauga, ON, Canada). Reverse transcription of the RNA was performed according to the procedure provided by the manufacturer (Expand Reverse Transcriptase Kit, Roche, Laval, PQ, Canada) and exons 20 – 28 (primer sequences Table 1) of the VWF cDNA were PCR amplified; a region spanning the putative mutation site. The amplified product was electrophoresed through a 1% agarose gel. The bands of interest were excised from the agarose gel, purified using the QIAquick Gel Extraction Kit, (QIAGEN, Mississauga, ON) and the cDNA was sequenced directly on an ABI model 373 automated sequencer (University Core DNA & Protein Services DNA Sequencing Laboratory, University of Calgary, Calgary, Alberta, Canada). To identify rare transcripts, the patient’s cDNA was ligated into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and 48 of the resulting colonies were analyzed by direct sequencing. The patient’s VWF cDNA was also PCR amplified and sequenced (Mobix Lab, McMaster University, Hamilton, Ontario, Canada) in the following segments: exon 16 – 21, exon 28 – 31, exon 30 - 36, exon 35 – 41, exon 40 – 52. A normal control platelet mRNA sample was treated in the same fashion.

Expression studies

The VWF expression vector pCIneoVWFES (kindly provided by Dr. P. Kroner, Medical College of Wisconsin, USA) consists of the full-length VWF cDNA inserted into the expression vector pCIneo (Invitrogen, Carlsbad, CA). A unique SalI restriction site was created by Dr. Kroner in exon 27 at codon 3608 of the
VWF gene. This SalI site in conjunction with a XbaI site at codon 357 was used to excise the 5’ end of the VWF gene which was subsequently subcloned into the multi-cloning site of Bluescript to produce the vector BluescriptVWFXbal-SalI. To introduce the G→A transition at nucleotide 3538, site-directed mutagenesis was performed on BluescriptVWFXbal-SalI using the overlap-extension procedure of Ho et al. 23 (primers listed in Table 1) and the resulting fragment was subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA). To create the constructs lacking exon 26 or exons 23/26, the patient's cDNA was amplified from exon 20 to exon 28 using the primers VWF-HS-ex20nt85-D and SalSDMex28-U and the amplified products were subcloned into pCR 2.1; all pCR 2.1 inserts were sequenced to verify the integrity of the amplified VWF gene. The fragments were excised from pCR 2.1 with BamHI and SalI (located in VWF cDNA at 2717 and 3608 respectively) and ligated back into BluescriptVWFXbal-SalI to produce the vectors BluescriptVWF1180R, BluescriptVWFdel26 and BluescriptVWFdel23/26. Finally, the XbaI to SalI fragments, were ligated back into the expression vector pCIneoVWFES to generate the cDNA expression vectors pCIneoG1180R, pCIneodel26 and pCIneodel23/26. All of the final constructs were sequenced to verify the integrity of the products.

COS-7 cells (ATCC, catalogue #CRL-1651) were cultured in Dulbecco modified Eagle medium (DMEM) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (vol/vol) fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells in the log phase of growth were used to seed tissue culture dishes such that the cells were approximately 50% confluent the following
day. These cells were transfected using the calcium phosphate technique with a total of 20 µg of DNA: 3.2 µg of βgal reporter construct, 11.8 µg of calf thymus DNA and 5 µg of the mutant plasmid or 5 µg of the wild-type plasmid (pCIneoVWFES) to yield homozygous mutant or wild-type genotypes respectively. The mutant and wild-type plasmids were also transfected in various ratios to yield a heterozygous genotype (wild-type:mutant ratios, 75:25, 50:50, 25:75). Forty-eight hours post-transfection, media was collected and cells were lysed using a cell lysis solution supplied in the Galacto Light Plus reporter gene assay kit from Tropix (Bedford, MA). The transfection efficiency was determined for each experiment by measuring the βgal activity from the βgal reporter transcript (that was co-transfected with the pCIneo plasmid) using a Berthold Lumat LB 9501 luminometer (Fisher Scientific Springfield, NJ) and the Galacto Light Plus reporter gene assay. The quantity of recombinant VWF:Ag present in the media and cell lysates was determined by ELISA using a Bio-Rad plate reader model 3550 (Mississauga, Ontario) and a polyclonal goat anti-human VWF antibody (Affinity Biologicals, Hamilton, Ontario) and the quantity of recombinant VWF protein measured in each experiment was normalized according to its transfection efficiency. The standard curve for the ELISA was generated using CryoCheck normal human reference plasma (lot #7070) from Precision Biologicals (Dartmouth, Nova Scotia). Recombinant VWF:Ag from the media was concentrated by dialysis for subsequent multimer analysis.

Results
Phenotype

The index patient is a 16 year old female from the Phillipines with a longstanding history of excessive mucocutaneous bleeding. She has had recurrent, significant epistaxis since a young age resulting in hospital admission on one occasion and many emergency room visits. The nosebleeds are often accompanied by welling of blood from her eyes. Shortly after menarche, she was admitted to hospital with severe menorrhagia and was treated with blood transfusions, DDAVP and cryoprecipitate. Her father has a history of mild mucocutaneous bleeding, although never requiring intervention. No other immediate family members were affected. An extended family history was not available.

The index patient's bleeding time was 8 minutes (normal range 2.5 – 9.5 minutes), the PTT was prolonged at 43.2 sec (normal range 27.2 – 36.0 sec) and the INR was normal at 1.0. The mean VWF:Ag, VWF:RCo and FVIII:C levels are 0.13 U/mL, 0.12 U/mL and 0.15 U/mL, respectively, and the RIPA showed no response at 0.5 g/L of ristocetin. There was also a loss of the high molecular weight VWF plasma multimers (Figure 1). There is no discrepancy between the VWF:Ag and the VWF:RCo in the pre-DDAVP results, which may reflect a lack of sensitivity for these assays (especially the VWF:RCo) at lower values. However, thirty minutes post-DDAVP her plasma levels were: VWF:Ag 1.02 U/mL, VWF:RCo 0.59 U/ml and FVIII:C 1.54 U/mL. It would have been interesting to evaluate the VWF multimers post-DDAVP to see if they are consistent with a Type 2A phenotype, however, this was not done. Based on this information, we reclassified the patient with Type 2A VWD.
Sequence variation

Sequence analysis in the index case revealed a putative mutation at 3538 G>A (Figure 2). Aside from common polymorphisms, no other sequence variations were observed in exons 18 – 52, introns 22, 23, 25, 26, nor in the flanking intronic sequence of any of the other introns. This change was also observed in the affected father, but was not present in the unaffected mother or brother. This change is located within the D3 domain of the mature VWF subunit, an area critical for proper multimerization.

RNA analysis for the 3538 G>A sequence variation

The 3538 G>A sequence variation is located at the last nucleotide of exon 26 and to examine its effect, RNA isolated from the platelets of the index case and a normal control were reverse transcribed and PCR amplified. When exon 20 – 28 was amplified, three VWF cDNA products (984, 825 and 684bp) were observed in the patient, but only one (984 bp) was identified in the normal control (Figure 3). The 984 bp band observed in the normal control and the patient revealed normal sequence; the 3538 G>A transition was not observed in the patient's cDNA (Figure 4a). Sequence analysis of the 825bp band from the patient revealed in-frame skipping of exon 26, plus a missense mutation P1127R caused by the last nucleotide of exon 25 creating a codon with the first 2 nucleotides of exon 27, instead of exon 26 (CCC to CGG) (Figure 4b and 4c). Sequence analysis of the patient-specific 684 bp band again showed in-frame skipping of
exon 26 with P1127R, plus in-frame skipping of exon 23 with no additional amino acid changes (Figure 4d and 4e). There were no differences noted between the patient and normal control for the RT-PCR products derived from exons 16-21, 28-31, 30-36, 35-41, or 40-52 (Figure 3b-d). Therefore, the patient is making three VWF transcripts: one normal transcript, one with skipping of exon 26 plus P1127R and one with skipping of both exons 23 and 26 plus P1127R. Because the 984bp band does not appear to contain the 3538 G>A transition, and the only other products identified are the exon skipped products, it appears that the allele with the transition mutation does not produce appreciable amounts of the G1180R product; instead, the transition results primarily in exon skipping. Although we observed homozygosity for ‘G’ at nucleotide position 3538, it is possible that there are a small proportion of transcripts containing the mutant ‘A’, undetectable by our sequencing methods. Approximately 50% of the cDNA products that were inserted into pCR 2.1 were missing exons 23 and 26, 33% were wild-type and 17% were missing exon 26.

**Expression and characterization of recombinant VWF:**

To determine the effect of exon skipping on VWF structure and function, the patient’s mRNA was amplified and the three spliced products (one normal and two exon-skipped transcripts) were introduced into the pClneoVWFES expression vector containing the full-length VWF cDNA. The G1180R mutation was also examined to determine if this amino acid substitution could impair VWF
synthesis and multimerization. The expression vectors pCIneoVWFES (wild-
type), pCIneoG1180R, pCIneodel26 (exon 26 skipped) and pCIneodel23/26
(exons 23 and 26 skipped) were transiently transfected into COS-7 cells alone or
together at various ratios (wt:mutant; 100:0, 75:25, 50:50, 25:75, 0:100) to
generate wild type, heterozygous and homozygous mutant genotypes. To
determine if the mutant recombinant protein was retained within the cell or was
efficiently secreted, VWF:Ag levels were assayed in cell lysates and in the
conditioned media using an ELISA and the data are presented in Figure 5.
Secretion of rVWF protein from the heterozygous and homozygous G1180R
transfections was marginally decreased by 14% and 19% respectively, when
compared to the secretion of the wild-type protein (not statistically significant,
p=0.36 for media, p=0.10 for lysate). However, the exon skipped transcripts
resulted in a significant reduction of secreted rVWF protein. Secretion of protein
missing exon 26 was reduced by 66% and 93% for heterozygous and
homozygous mutant transfections respectively. Secretion of rVWF protein
missing exons 23 and 26 was even more disrupted with secreted protein levels
being reduced by 78% and essentially 100% for the heterozygous and
homozygous mutant transfections respectively. For all of the experiments, the
decrease in recombinant protein found in the media corresponded to an increase
in intracellular retention. These results illustrate the dominant-negative nature of
these aberrantly spliced proteins. Furthermore, these results demonstrate that
the mutant cDNA with both exons 23 and 26 spliced out causes more retention of
the VWF protein compared to the construct missing only exon 26.
To determine the effect of the mutations on VWF multimers, conditioned media from COS-7 cells for the homozygous wild-type, homozygous mutant and 50:50 heterozygous transfections was concentrated by dialysis and analyzed by SDS-agarose gel electrophoresis (Figure 6). All sizes of multimers were observed for the G1180R recombinant protein, suggesting that this mutation does not impair multimerization. A full range of multimers were also observed in the media from the heterozygous transfections (involving wild-type and exon-skipped cDNA constructs). Only the lowest molecular weight multimers are observed from recombinant protein missing exon 26 (homozygous) and the rVWF missing both exons 23/26 (homozygous) indicating that the missing cysteine residues are imperative in the formation of multimers.

DISCUSSION

In this report, we present a novel single base pair change at the last nucleotide of exon 26 that acts as the molecular basis for a young woman’s Type 2A VWD. We have shown that this mutation results in skipping of not only exon 26, but also of exon 23 in a population of mutant transcripts. To the best of our knowledge, this is the first demonstration of multiple, non-adjacent exon skipping as the pathogenic basis for an inherited disease.

Splicing is a complicated process that involves the precise identification and joining of exons and removal of introns. For this to happen, five small nuclear (sn) RNAs (U1, U2 and U4-6) and more than 60 polypeptides must
correctly assemble into a large multicomponent ribonucleoprotein complex known as the spliceosome.\textsuperscript{24, 25} The spliceosome recognizes conserved, consensus sequences including the 5’ (donor site) and 3’ (acceptor site) splice sites that flank mammalian exons. In addition to the donor and acceptor sites, the last 2 nucleotides of an exon are also semi-conserved; the second last is an “A” 64% of the time, and the last is a “G” 73% of the time. It is conceivable that changes to the last nucleotides of an exon might lead to aberrant splicing due to the donor site not being recognized by the splicesome complex. The mechanism by which the sequential pairing of splice sites is accomplished is not known, however the assembly of the spliceosome, while the RNA transcript is still being transcribed, is presumed to play a major role in the orderly pairing of appropriate splice sites.\textsuperscript{26, 27} It is known that the splicing machinery associates with exons in a 5’ to 3’ order as they are synthesized, however, the covalent joining of exons does not necessarily proceed in a 5’ to 3’ direction due to inherent differences in rates of splicing catalysis between each exon pair.\textsuperscript{28}

In order to understand the transcript that is missing exon 26, we must examine the possibility that a change in the last nucleotide of an exon could lead to aberrant splicing. There are many precedents in the literature for this, all based on the idea that mutations at this site would lead to inactivation of the 5’ donor splice site, and pairing of the donor site from one exon with the acceptor site of another, resulting in skipping of the adjacent exon. This has been described in a number of inherited diseases.\textsuperscript{12 – 19} The change in the last nucleotide of exon 26 is the only putative mutation identified in our patient, after
sequencing of the coding regions of exons 18-52 with flanking introns, and the vast majority of the introns surrounding the skipped exons (intron 22 was not completely sequenced because of its large size and the very low possibility that changes so far from the mutant exon might lead to exon skipping). In the patient’s cDNA we only observed the ‘G’ at nucleotide position 3538, although it is possible that there are small, unappreciable amounts of the mutant ‘A’ allele present, which would produce the missense mutation G1180R. However, the expression studies on G1180R clearly show that it does not produce a Type 2A VWD phenotype and is not likely contributing to the patient’s phenotype. This phenomenon, of a putative missense mutation leading instead to a splicing mutation, has recently been described in a case of severe hypofibrinogenemia.\textsuperscript{29} Therefore, in our patient, in order to conclude that the single nucleotide change in the last nucleotide of exon 26 is the molecular basis of this patient’s Type 2A VWD, we must consider how this change leads not only to skipping of exon 26, but also to skipping of exon 23 in some transcripts.

There is strong evidence to support the theory that order and speed of splicing are important determinants of mRNA transcripts with exon skipping.\textsuperscript{30-33} Despite the fact that the spliceosome associates with the transcript as it emerges from the polymerase, the joining of exons does not occur in order from the 5’ end to the 3’ end of the transcript. Introns with strong splicing consensus sequences are often removed more efficiently than introns with weaker consensus sequences; thus, some exons located downstream will be joined together before slower splicing, upstream exons. Takahara et al\textsuperscript{30} describe a COL5A1 acceptor
site mutation causing Ehlers-Danlos Syndrome Type 1, that results in the production of a number of transcripts, including one missing exon 5 and 6, one missing only exon 5 and other products formed by the activation of cryptic acceptor sites within exon 5. They, as well as other investigators,\textsuperscript{31, 33} conclude that the order and speed of splicing can be responsible for different transcripts. Splicing mutations of fast-spliced introns often lead to exon skipping however, splicing mutations of slower spliced introns often lead to cryptic splice site activation and intron inclusion.\textsuperscript{31, 33} In this patient, the substitution is located in the last nucleotide of exon 26, leading to the pairing of the donor site from intron 25 with the 3' acceptor site from intron 26. Because the donor and acceptor site of intron 26 have a strong consensus splicing sequence score, and no products that could result from the activation of a cryptic splice site were observed, we hypothesize that intron 26 is a fast-spliced intron. Possible cryptic donor sites were examined with the program NNSplice (\url{http://www.fruitfly.org/seq_tools/splice.html}), however, only the natural donor site had a high score.

In the Takahara paper\textsuperscript{30}, a transcript is described which is missing 2 adjacent exons; this is explainable by speed and order of splicing. However, it is difficult to apply this argument to the splicing of 2 non-adjacent exons, as in our patient's transcript with both exon 23 and 26 skipped, thus we must consider other potential mechanisms for this aberrant splicing. It is possible that the initial event of skipping exon 26 disrupts the subsequent upstream splicing of exon 23. Exonic sequences have been recently recognized as important controllers of
alternative splicing. Exonic splicing enhancers (ESE) act as binding sites for specific serine/arginine-rich (SR) proteins.\textsuperscript{34} These SR proteins recognize RNA motifs and act to enhance splicing by either directly recruiting the splicing machinery or by antagonizing the action of nearby silencer elements.\textsuperscript{35,36} Exonic splicing silencers (ESS) are not as well understood but seem to work by interacting with negative regulators, often belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family.\textsuperscript{37} In our patient, the change at the last nucleotide of exon 26, leading to the skipping of that exon, results in the destruction of an ESE for SRp40 (analysis done using the web based program ESEfinder\textsuperscript{38} http://exon.cshl.edu/ESE/) It is possible that the correct splicing of exon 23 is dependent on that ESE and that its destruction leads to exon 23 skipping in some transcripts.

A second proposed model for exon 23 skipping is the interaction of G-rich and C-rich elements found in introns 22 and 23 that might be facilitated by the exon 26 mutation.\textsuperscript{39} This interaction could result in the formation of a hair-pin structure which would cause looping out of exon 23 and result in the juxtaposition of the donor splice site from intron 22 with the acceptor splice site of intron 23. The secondary structure of VWF mRNA was examined using the program Mfold\textsuperscript{39} (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) and sequences from introns 22 and 23 were found to be complementary making the above proposal plausible.

The presence of two mutant transcripts and one wild type transcript in our patient is key to understanding the dominant-negative effect of the mutant
subunit on the correct multimerization of VWF. Skipping of exon 26 causes the in-frame deletion of 54 amino acids including 9 cysteine residues. It also causes the non-conservative amino acid change P1127R; however, the skipping of two critical exons likely contributes more significantly to the pathogenesis of the phenotype in this case. Skipping of exon 23 causes an in-frame deletion of 48 amino acids with the further loss of 3 cysteines. Exons 23 and 26 both encode regions of the D3 domain at the N-terminus of the mature VWF subunit which plays a critical role in the multimerization of the VWF protein. In light of the location of the skipped exons, deletion of these 12 cysteines would be expected to have disastrous effects on the multimerization of the patient’s VWF protein.

The expression studies clearly show that the cDNA missing both exon 23 and 26 results in more intracellular retention than the exon 26 skipped construct, however both mutant constructs exhibit a dominant-negative effect on the VWF subunits translated from the normal cDNA, offering an explanation for the severity of the patient’s phenotype. The probability that a dimer would contain a mutant subunit is 75%, assuming dimerization in the ER is random. Most of these mutant dimers will be retained within the cell but some dimers containing a mutant subunit may be processed into multimers. One possible explanation for how this might lead to abnormal multimerization is that these mutant subunits may act as reducing-ends during multimer formation; thus additional dimers can only be added to one end of the multimer not both. This would result in a reduction in the size of secreted VWF multimers, which is consistent with the patient’s plasma multimer profile. An unexpected finding in our study was that
all sizes of multimers were observed in the heterozygous transfection experiments using wild-type and exon-skipped recombinant cDNAs. This discrepancy between the multimer pattern of the heterozygous rVWF proteins and the patient’s plasma multimers may well reflect the consequence of several limitations of the recombinant expression system, and is a phenomenon that has been documented in previous similar studies.  

Examination of the Intron Sequence Information System (ISIS) revealed 109 genes that demonstrated in-frame exon skipping, in one or more exons; however, none of these genes demonstrated skipping of non-adjacent exons. This is the first report that we are aware of in which a change in the last nucleotide of an exon is shown to cause skipping of two non-adjacent exons, and the molecular mechanism demonstrated in this case of Type 2A VWD raises interesting questions about the mechanics of splicing in the VWF gene. Furthermore, it highlights the importance of examining putative mutations at the mRNA level.

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Table 1: Primers for RT-PCR, Sequencing and Site Directed Mutagenesis

<table>
<thead>
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<th>Primer</th>
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Table 1: F=forward primer, R=reverse primer. Primer sequences in italics and underlined were used to perform reverse transcriptase reactions, underlined nucleotides are those that are different from the pseudogene, and bold nucleotides indicate base substitutions for mutagenesis reaction. Location indicates first nucleotide of primer with the numbering according to Mancuso et al., except for the location of 22F which is shown as the distance from the start of exon 23.
Figure Captions:

Figure 1a: Patient’s VWF multimer profile
Autoradiograph of plasma VWF multimers for normal plasma (lane 1), propositus’ plasma (lane 2) and a Type 2A VWD control plasma (lane 3). Plasma samples were electrophoresed through a 1.6% SDS-agarose gel and multimers were visualized by chemiluminescence. The highest molecular weight multimers and all the satellite bands are absent from the propositus’ plasma.

Figure 1b: Densitometry of VWF multimers
Densitometry of VWF multimers for normal plasma (top lane), propositus’ plasma (middle lane) and a Type 2A VWD control plasma (bottom lane) – densitometry was performed on the same 1.6% SDS-agarose gel as in Fig 1a. The values shown are for the area under the curve in square pixels. For the normal, the total number of pixels is 19945, with 4284 pixels or 21% above the 9th multimer band. For the propositus the total is 9509, with 1129 or 11% above the 9th multimer band. For the Type 2A VWD control the total is 13740, with 737 or 5% above the 9th multimer band.

Figure 2: DNA sequence analysis of exon 26.
Sequence from the propositus reveals a G>A transition at nucleotide 3538 which is the last nucleotide of exon 26. The propositus is heterozygous for this transition as is indicated by the double chromatogram peak.
Figure 3: Amplification of cDNA from the propositus and a normal control

cDNA from platelet RNA was amplified from the propositus and a normal control. Panel a: Amplification of exons 20-28 resulted in one product of 984 bps in the normal control (lane 2); however, three bands (984 bp, 825 bp and 684 bp) were observed in the patient’s amplified product (lane 1), corresponding to the correct product, and products missing exon 26 and exons 23/26, respectively. The mRNA corresponding to the remaining mature VWF peptide was also amplified in different segments; however, no difference was observed between the normal control and patient. Panel b: amplification of exons 16-21 generated a 705 bp product (lane 1 is H2O, lane 2 is normal control cDNA and lane 3 is the patient’s cDNA). Panel c: amplification of exons 28-31 generated a 1371 bp fragment (lane 4 H2O, lane 5 normal control, lane 6 patient), and amplification of exons 30-36 produced a 1103 bp fragment (lane 1 H2O, lane 2 normal control, lane 3 patient). Panel d): amplification of exons 35-41 generated a 1216 bp fragment (lane 4 H2O, lane 5 normal control, lane 6 patient) and amplification of exons 40-52 produced a 1620 bp fragment (lane 1 H2O, lane 2 normal control, lane 3 patient).

Figure 4: cDNA Sequence from the three bands amplified in the patient for exon 20-28

Panel a): Sequence from 984 bp band showing homozygosity for the wild-type nucleotide (G) at nt 3538, codon 1180. Panel b): Sequence from the 984 bp
band shows that exon 25 is spliced to exon 26. Panel c): The 825 bp band has exon 26 spliced out, therefore exon 25 is spliced to exon 27. The reading frame of the message remains intact. However, an amino acid change results (Pro to Arg) where the last nucleotide of exon 25 pairs with the first two nucleotides of exon 27. Panel d): Sequence from the 984 bp band shows that exon 22 is spliced to exon 23. Panel e): Sequence from the 684 bp band revealed that exon 23 was skipped in addition to exon 26. This sequence demonstrates the in-frame splicing of exon 22 to exon 24. No amino acid changes result from the in-frame exon skipping.

Figure 5: Intracellular retention of recombinant mutant VWF.

Panel A: Relative concentrations of the wild-type G1180, mutant R1180 and heterozygous G1180R proteins in COS-7 supernatants and cell lysates (mean +/- SEM) (N=3).

Panel B:
The concentration of rVWF protein secreted into the media by COS-7 cells was decreased in transfections using the pClneodel26 plasmid (solid black bar) when compared to wild-type transfections. As the quantity of rVWF secreted into the media decreased with increased concentrations of mutant plasmid, the amount of protein found retained within the cell increased (hatched dark bar). Likewise, the concentration of rVWF protein secreted into the media from transfections using the pClneodel23/26 plasmid was also decreased (solid white bar); this double mutation showed a trend towards increased intracellular retention (hatched white bar) when compared to the single mutation at the 75/25 and 50/50 transfection
ratios. When 100% pCIneodel23/26 was transfected into COS-7 cells, no protein was secreted into the media (mean +/- SEM) (N=3). The values from the heterozygous and homozygous mutant ELISAs are normalized to those of wild-type (setting wild-type levels at 1).

Figure 6: Autoradiograph of recombinant VWF multimers.

rVWF protein secreted into the media was concentrated and multimer analysis was performed. Panel A: Lane 1, 100% wild type; lane 2, heterozygous (cotransfection of wild type and pCIneoG1180R plasmid), lane 3, 100% G1180R mutant. The G1180R mutant protein does not result in a loss of HMW multimers. Panel B: Lane 1, 100% wild type; lane 2, heterozygous (cotransfection of wild type and pCIneodel26 plasmid); lane 3, heterozygous (cotransfection of wild type and pCIneodel23/26 plasmid); lane 4, homozygous pCIneodel26 mutant; lane 5, homozygous del23/26 mutant; lane 6, 100% wild type. The homozygous transfections both show the presence of only the lowest molecular weight multimers, whereas the heterozygous transfections show a normal multimeric pattern.
Figure 1a

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Figure 1b:
Figure 2
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Figure 3

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a. 984 bp  
825 bp  
684 bp

b. 705 bp

c. 1371 bp  
1103 bp

d. 1620 bp  
1216 bp
Figure 4
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Exon 26     Exon 27

CAG   GAG
Gln     Glu

Exon 25     Exon 27

CGG
Arg

c.

Exon 22     Exon 24

CAG    GTG
Gln       Val

a.

b.

c.

d.

e.
Figure 5

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A.

B.
Figure 6

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A.
A Novel Type 2A von Willebrand Factor Mutation Located at the Last Nucleotide of Exon 26 (3538G>A) Causes Skipping of Two Non-Adjacent Exons

Paula D James, Lee A O'Brien, Carol A Hegadorn, Colleen R Notley, Gary D Sinclair, Christine Hough, Man-Chiu Poon and David Lillicrap

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