Rapid molecular diagnosis of von Willebrand disease by direct sequencing. Detection of 12 novel putative mutations in VWF gene

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Summary

Molecular diagnosis of von Willebrand Disease (VWD) is particularly complex. The autosomal von Willebrand factor gene (VWF) is large and highly polymorphic, and there is a highly homologous (>96%) partial pseudogene in chromosome 22. Because of these difficulties, application of molecular study of VWD to the clinical routine has been considerably delayed. Recent advances in sequencing technology and bioinformatics could convert direct sequencing of the complete VWF into a routine diagnostic tool for VWD, which is especially desirable in types 1 and 3. This study describes a highly optimized procedure in which all the coding and intronic flanking regions of VWF are amplified under identical thermocycling parameters in a ready-to-use PCR plate format. The entire sequencing procedure, from blood extraction to mutation identification, can be done within 24 hours, resulting in a simple, versatile, cost-effective strategy with little hands-on time requirements. To validate the method, we performed full-length VWF sequencing of 21 index cases including seven of each VWD type. A total of 30 VWF genetic variations were identified. Twelve of these sequence variations are new, including four missense, one nonsense, one insertion, the first insertion-deletion described in VWF, and 5 potential splice site mutations. To our knowledge, this is the fastest and most efficient protocol designed to date for complete sequencing of the VWF coding region in the molecular diagnosis of VWD.

Keywords
Automated DNA sequencing, molecular diagnosis, mutation analysis, von Willebrand disease, von Willebrand factor

Introduction

Von Willebrand disease (VWD) is the most common congenital bleeding disorder in humans, occurring in around 1% of the population. It is caused by a quantitative (types 1 and 3) or qualitative (type 2) defect of von Willebrand factor (VWF), a multimeric glycoprotein that plays an important role in primary haemostasis, promoting platelet adhesion at sites of vascular injury, as well as serving as a carrier protein for factor VIII (1). Clinical diagnosis of VWD, particularly type 1, can be complex because several variables (e.g. blood group, age, sex, exercise, oral contraceptive use) can influence VWF plasma levels (2–4); substantial variations are often evident during serial sampling in patients suspected of having the disease (5, 6). Accurate diagnosis of type 2 variants is also crucial, since the VWF protein is dysfunctional in these patients. Thus, exogenous VWF concentrates are typically administered rather than desmopressin, which would release endogenous dysfunctional VWF (7). Desmopressin in induces the release of stored VWF from the tissues into the bloodstream and it considered the best treatment for type 1 VWD (8).

Molecular diagnosis of VWD is particularly hampered by the following factors: the large size of VWF gene (VWF) (178 kb), which is comprised of 52 exons; presence of a highly homologous (>96% homology) partial pseudogene in chromosome 22; the fact that VWF is highly polymorphic; and, lastly, the diagnosis of type 1 VWD is linked to VWF only in around 41% to 70% of families (9, 10). Fortunately, many type 2 mutations are located in exons 18 to 24 (type 2N) and in exon 28 (types 2A, 2B, and 2M), which makes DNA sequence analysis straightforward in these cases (11), and the molecular defect in many type 2 mutations has been described (12). However, types 1 and 3 VWD mutations are not restricted to specific exons; hence, the study of these mutations requires analysis of all the essential VWF regions. Nonetheless, DNA sequencing of the complete VWF coding region has not yet become routine, and mutations in type

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1 VWD are notoriously underrepresented in the International Society on Thrombosis and Haemostasis (ISTH) VWF database. Mutations responsible for type 1 VWD (70% of families) comprise only 31% of the records, in contrast to type 2 mutations (15% to 20% of cases), which are over-represented with 41% of the records. This imbalance was even more evident in the contents of the database prior to 2007, when there were only 24 entries for type 1 patients. Three recently published multicenter studies from the United Kingdom (13), the European Community (14), and Canada (15) have contributed 135 additional type 1 records.

In the light of these data, it is clear that the development of simpler, more comprehensive and precise molecular diagnostic tools is essential to support the clinical diagnosis of VWD. Numerous methods have been described to detect sequence variations in large genes, ranging from the direct sequencing approach to a variety of screening techniques. Among others, comprehensive analysis of VWF by denaturing high-performance liquid chromatography (dHPLC) (11), as well as direct sequencing (15) are both effective for mutation detection in VWD. Taking advantage of the innovations in DNA sequencing technologies, the aim of this study was to address the need for accurate, high-throughput genotyping of VWF (11), as well as direct sequencing (15) are both effective for mutation detection in VWD. Taking advantage of the innovations in DNA sequencing technologies, the aim of this study was to address the need for accurate, high-throughput genotyping of VWF by denaturing and simplifying several processes required for its direct sequencing. To incorporate this powerful technique into our laboratory’s routine practice, we have proven its utility by full-length VWF sequencing in 21 index cases, identifying a total of 30 VWF genetic variations, 12 of which are new.

Materials and methods

Patients and DNA extraction

Samples were collected and clinical data compiled from 48 patients in 21 families of several origins affected by different VWD types. Complete sequencing of VWF was performed in 21 index cases (7 families with type 1, 7 with type 2, and 7 with type 3 VWD). Families diagnosed at Vall d’Hebron Hospital as having possibly or possibly having type 1 VWD were included, and index cases met the ISTH criteria (16): low plasma VWF levels with a normal multimeric VWF pattern, significant bleeding symptoms, and at least one additional family member with type 1 VWD or significant bleeding symptoms. Laboratory data for index cases (VWF:Ag, VWF:RCo, FVIII:C and blood group) are presented in Table 1. All index cases showed a multimeric VWF pattern consistent with the corresponding VWD type classification. The study was approved by the Ethics Committee of Vall d’Hebron Hospital, and all participants provided their informed consent for enrollment.

Samples of peripheral blood from patients and family members were collected into tubes containing ACD or EDTA as anticoagulant. Genomic DNA was isolated from 300 µl of blood with the FlexiGene DNA Kit (Qiagen, Duesseldorf, Germany).

Microsatellite analysis

A multiplex fluorescent PCR recently described by our group (17) comprising three VWF intragenic tetranucleotide repeats (STR-1, STR-2 and STR-3) and one in promoter STRs (WPA), was applied to genomic DNA samples. All participating individuals (index cases and relatives) were successfully genotyped for all markers; hence, there were no missing values.

Primer design

Primers were automatically designed with the aid of Exon Primer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) (18). The cDNA sequence of VWF (GenBank no. NM_000552) and the corresponding genomic sequence (GenBank no. NC_000012.10; range 592830: 6104097) were used as input. This program provided the list of forward and reverse primers used to amplify all exons except for the region homologous to the partial pseudogene in chromosome 22 (exons 23 to 34). To amplify these exons, we designed specific primers taking into account the precise differences between the VWF genomic sequence and the pseudogene, to ensure highly specific amplification. Moreover, all the primers designed were aligned against the VWF region according to dbSNP (Build 128) to corroborate the absence of SNPs in primer binding sequences that could result in preferential or single allele amplification, and lead to missing a mutation. Only primer VWFEE28–1B (GTGGGAA-TATGGAGTCTCRTTG) was degenerated at position 18 to avoid unbalanced allele amplification. After examining different primer combinations, the optimized amplification and sequencing primers obtained are those presented in Table 1 of the Supplementary material (available online at www.thrombosis-online.com).

VWF amplification

Primers and MgCl2 are pre-dispensed in a ready-to-use microplate format (2 patients can be amplified on each microplate). The PCR solution contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl2, 200 µM dNTPs, 2 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.75 µM of each primer, and 100 ng of DNA in a total volume of 25 µl. After initial denaturation at 94°C for 3 minutes (min), 34 cycles of 94°C for 20 seconds (sec), 62°C for 30 sec, and 72°C for 1 min were performed, followed by a final extension at 72°C for 3 min. To obtain good yield in the established PCR conditions (Fig. 1), primer concentration was adjusted to 1.25 µM for amplification of exons 27 and 32, and to 2.5 µM for exons 14 and 29-30. Dimethylsulfoxide (DMSO) at 4% was added to the PCR mix for exon 15 because it is located in a GC-rich genomic region refractory to PCR amplification (19).

DNA sequencing

An aliquot of each of the 47 amplification reactions per patient was transferred to a fresh microplate, enzymatically purified by ExoSAP-IT treatment (USB Corporation, Cleveland, OH, USA), and directly sequenced by dideoxynucleotide method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the primers listed in supplementary material Table 1. The resulting 55 sequencing reactions were analyzed by capillary electrophoresis in an ABI PRISM 3100-Avant (Applied Biosystems). The basic protocol includes unidirectional sequencing of each exon, and the putative mutations detected were always validated on a second independent PCR product using both forward and reverse primers to confirm the sequence variation. In addition, all available
**Table 1: Summary of molecular data.**

<table>
<thead>
<tr>
<th>Index case/ family number</th>
<th>VWD type</th>
<th>[VWF:Ag], [VWF:RCo], [FVIII:C]</th>
<th>Blood group</th>
<th>Mutation type</th>
<th>Genic region</th>
<th>Nucleotide change</th>
<th>AA change</th>
<th>Domain</th>
<th>STRs genotype</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 1</td>
<td>O</td>
<td>Missense</td>
<td>E28</td>
<td>4135C&gt;T</td>
<td>R1379C</td>
<td>A1</td>
<td>19–6–12–14 (g) 22–7–16–17</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 1</td>
<td>O</td>
<td>Missense</td>
<td>E15</td>
<td>1781C&gt;G&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A594G</td>
<td>D2</td>
<td>17–13–14–10 20–6–12–15</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41 1</td>
<td>O</td>
<td>Missense</td>
<td>E40</td>
<td>6932G&gt;A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R2311H</td>
<td>B1</td>
<td>19–7–14–15 20–13–14–18</td>
<td>Mutation not found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 1</td>
<td>O</td>
<td>Missense</td>
<td>E36</td>
<td>6187C&gt;T</td>
<td>P2063S</td>
<td>D4</td>
<td>21–7–14–15 19–11–14–15</td>
<td>Also described as polymorphism in ISTH database</td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 1</td>
<td>O</td>
<td>Missense</td>
<td>E19</td>
<td>2446C&gt;T</td>
<td>R816W</td>
<td>D’</td>
<td>21–6–12–18 21–6–12–19</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 2M</td>
<td>A</td>
<td>Missense</td>
<td>E28</td>
<td>4225G&gt;T&lt;sup&gt;*&lt;/sup&gt;</td>
<td>D1</td>
<td>D3</td>
<td>22–7–15–17 19–6–12–19</td>
<td>Homozygous mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 2A</td>
<td>A</td>
<td>Missense</td>
<td>E28</td>
<td>4102C&gt;T</td>
<td>R1374C</td>
<td>A1</td>
<td>19–6–12–17 21–7–15–14</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 2A</td>
<td>A</td>
<td>Missense</td>
<td>E28</td>
<td>4102C&gt;T</td>
<td>R1374C</td>
<td>A1</td>
<td>19–6–12–17 21–7–15–14</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 3</td>
<td>O</td>
<td>Missense</td>
<td>E6</td>
<td>546G&gt;A&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>S182S</td>
<td>D1</td>
<td>22–11–14–17 19–7–14–15</td>
<td>Compound heterozygous in trans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 3</td>
<td>A</td>
<td>Missense</td>
<td>E5</td>
<td>7730–1G&gt;C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>21–7–14–16 21–7–14–16</td>
<td>Homozygous. Non-consanguineous descent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 3</td>
<td>A</td>
<td>PSSM</td>
<td>E5</td>
<td>7082–2A&gt;G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>L150P</td>
<td>D1</td>
<td>20–11–14–16 19–7–14–16</td>
<td>Compound heterozygous in trans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62 3</td>
<td>A</td>
<td>INDEL</td>
<td>E5</td>
<td>375–376delG-T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>L1382L</td>
<td>D4</td>
<td>19–7–14–16 19–7–14–16</td>
<td>All three homozygous mutations. Consanguineous descent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Novel putative mutations; PSSM: potential splice site mutation; g: haplotype not established; AA, amino acid.
family members were sequenced for the putative mutation to confirm family transmission.

Sequence analysis
Sequences obtained by the Sequence Analysis software (Applied Biosystems) for each patient (all exons and twenty intronic base pairs flanking both sides of each exon) were assembled in batch mode and aligned with the consensus wild-type VWF sequence (GenBank no. NC_000012.10) using the SeqScape® v2.1.1 software (Applied Biosystems). For this purpose we designed a SeqScape® template (available upon request) including the annotated VWF sequence to automatically discriminate between unknown variants and known polymorphisms (64 polymorphisms in the coding region of VWF according to dbSNP Build 128). This template also includes a library containing the pseudogene sequence to avoid misdiagnosis owing to non-specific amplification of the pseudogene.

The predicted impact of the novel missense mutations described was assessed with PolyPhen (http://genetics.bwh.harvard.edu/pph/) and the potential splice site mutations with NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/). Because of the limitations of in silico tools (20) a second program was used to perform the analysis: Sort Intolerant from Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html) for missense mutations and Human Splicing Finder (HSF; http://www.umd.be/SSF) for potential splice site mutations.

Results
An automated, high-throughput procedure to directly sequence VWF has been designed and optimized as a tool for molecular diagnosis of VWD. For this purpose, thermocycling parameters and primer design were greatly optimized to obtain high-yield and specificity amplification under an identical MgCl₂ concentration for all PCR products in a ready-to-use PCR plate (Fig. 1). A total of 47 amplification reactions per patient are necessary to cover the 52 exons (Fig. 1). Using a four-capillary instrument, the total time from blood sample collection to mutation identification is 24 hours (h) per patient with only 6 h of hands-on time. All the individuals enrolled (index cases and relatives) were systematically genotyped for four STR polymorphisms using the single-tube automated fluorescent technique recently described by our group (17).

Based on the sequencing strategy described above, and to validate the method for routine used in molecular diagnosis, we performed full-length VWF sequencing in 21 index cases, including seven of each VWD type. A total of 30 VWF genetic variations (or 25 considering a gene conversion as a single mo-
Mutations in type 1 patients

We were unable to identify any genetic variation in three of the seven (42%) type 1 VWD patients studied. In the remaining four index cases, a single putative point mutation was identified per patient, two previously described and two novel mutations: R2311H (E40: 6932G>A) predicted as possibly damaging (1.82) and a frameshift indel, and five potential splice site mutations.

Table 1. Twelve of these genetic variations have been reported previously and 12 are new, with one (7082–2A>G) found in two patients. The novel sequence variations include four missense, one nonsense, one insertion, one insertion-deletion (indel), and five potential splice site mutations.

Mutations in type 2 patients

All the type 2A mutations have been previously described; the V1279F mutation, however, was described in a 2M patient (21). Because no other mutation was found along the coding region, together with the fact that the index case and relatives show a total absence of high-molecular-weight multimers (data not shown), the mutation should be classified as 2A. The 2M-type VWD patient (case 32) presented the novel putative mutation V1409S (4225G>T in exon 28) in compound heterozygous status with the previously described missense mutation, P2063S. Although it has been reported that such variations occur at a frequency of 0.25 in unaffected family members, our data seems to indicate a potential deleterious effect. Since index case 32 has a more severe phenotype than her mother and son, complete VWF sequencing of all three family members was performed to determine whether possible variations in the genetic background correlated with the differing clinical behavior. The novel missense mutation (4225G>T, V1409S) was identified in all three family members, and the second mutation (6187C>T, P2063S) was exclusively detected in the index case. This could be a reasonable cause for the clinical differences observed, although other explanations cannot be ruled out.

Mutations in type 3 patients

Seven index cases classified as type 3 were studied at the molecular level. We found a large number of genetic variations in only seven patients. Among the putative mutations identified in type 3 VWD index cases, the insertion, the frameshift indel, and two nonsense mutations (Table 1) have clear functional consequences due to the resulting truncated protein. Silent, missense, and intronic sequence variations were also identified. All potential splice site mutations were analyzed by in silico methods with NetGene2 and HSF software; results are summarized in Table 2. Index case 1 has three previously unreported mutations: one in the consensus acceptor splice site of intron 41 (7082–2G>A), one in the consensus donor splice site sequence of intron 50 (8155+3G>C), and the synonymous 546G>A (S182S) mutation at exon 6, which could potentially affect the donor splice site of intron 5, according to the NetGene2 and HSF predictions. Mutations 7082–2G>A and 546G>A were allelic.

Three families with documented consanguineous descent were also analyzed, and the index cases showed putative homozygous mutations as the expected cause of type 3 VWD. In index case 6, we found a homozygous insertion of two nucleotides (7664–7665insAG), which leads to a frameshift at valine 2556 and predicts the addition of seven aberrant amino acids be-

| Table 2: Summary of in silico analysis of novel missense and potential splice site variations. |
|-----------------|-----------------|-----------------|-----------------|
| Nuc. change     | AA change       | Domain          | PolyPhen prediction (score') | SIFT prediction (score) |
| 4225G>T         | V1409S          | A1              | Possibly damaging * (1.811) | Affect protein function (0.00) |
| 1781C>G         | A594G           | D2              | Benign (1.262)              | Affect protein function (0.02) |
| 6932G>A         | R2311H          | D1              | Possibly damaging (1.82)    | Affect protein function (0.00) |
| 6197A>G         | N2066S          | D4              | Possibly damaging (1.699)   | Tolerated (0.06) |

Phenotype
fore a premature stop codon is encountered. In index case 18, the exon 28 sequence revealed multiple homozygous mismatched bases. Analysis of this exon by alignment with the homologous region of the pseudogene revealed that gene conversion (involving six base changes) had probably taken place. The 3931C>T base change results in a stop codon, which would lead to premature termination of the protein. The maximal length of the gene conversion was 335 bp and the minimal length 271 bp. Lastly, in index case 62, a novel indel homozygous mutation (375–376delGTinsC) was encountered that causes a frameshift at glycine 125 and predicts the addition of 48 aberrant amino acids before a premature stop codon. Moreover, 2 additional new genetic homozygous variations were identified in this patient (Table 2).

Discussion

The gold standard for mutation detection is DNA sequencing, but for the large VWF, sequencing of the entire coding region has not yet become routine practice (22). Herein, we demonstrate that it is possible to directly sequence and analyze all the essential regions of VWF on a routine basis. In our standard procedure, DNA sequencing is undertaken in only one direction. Undoubtedly, alignment of both sequence directions would resolve nearly all artifacts, such as G/C compressions or other irregular mobilities related to the DNA secondary structure. However, our data from preliminary experiments using bidirectional DNA sequencing for all PCR products (not shown) indicate that the number of genetic variations (mutations and polymorphisms) detected is virtually identical to the number found with unidirectional sequencing, provided that the BigDye 3.1 Cycle Sequencing kit is used. Thus, only the putative mutations detected are validated on a second independent PCR product using both forward and reverse primers, as is stated in Material and methods. Additionally, when any unclear sequence alteration is detected, a reverse sequence entry is performed to confirm or rule out the change. The unidirectional sequencing option is a cost-effective approach.

To validate the method for routine use in molecular diagnosis, complete VWF gene sequencing was performed with the described sequencing strategy in a total of 21 index cases, including seven of each VWD type. Virtually all the predicted mutations in type 2 and 3 VWD were detected; all type 2 patients had a candidate mutation(s) that explained their phenotype and all type 3 patients also had a number of mutations that would potentially explain their deleterious nature. Because all the regions considered relevant for VWF expression are systematically sequenced, the undesirable masking effect caused by polymorphic variations observed with some screening techniques (11) is averted. Taking these data together, it is likely that we identified all the mutations in the coding region of the type 1 VWD cohort. Nevertheless, the putative mutation was detected in four (57%) of the seven type 1 index cases sequenced, a rate similar to reported values (14, 15). The difficulty in identifying the putative mutation in these cases may be attributable to the fact that our standard procedure for direct sequencing is unable to detect some mutations that have also been described as responsible for quantitative VWF defects, such as large deletions (23), mutations in the promoter region (15), and deep intronic mutations. Moreover, mutations in other genomic regions cannot be ruled out.

We detected 12 novel singular putative mutations in VWF, three of which were nonsense, small insertion, or indel changes. The deleterious mechanisms of these mutations are, in general, obvious given that they create premature termination codons leading to a frameshift and truncated VWF protein. As to the clinical significance of the putative novel missense mutations found, the predicted impact was assessed with PolyPhen and SIFT software, obtaining the scores summarized in Table 2.

Among novel mutations in type 1 patients, A594G was predicted to be a benign mutation by PolyPhen. Nevertheless, this mutation was considered to affect protein function when it was analyzed with SIFT based on sequence homology and the physical properties of amino acids (24). Interpretation of the results of in silico prediction programs should not be used to decide, without further evidence, whether the sequence variation found in a patient is or is not responsible for the disease (25). These limitations, together with the presence of multiple exonic polymorphisms that make interpretation of molecular analysis results difficult (7), indicate that expression studies in vitro of these mutations may be needed to clarify their deleterious nature.

Among novel mutations found in type 2 patients, the V1409S substitution (potentially responsible for the 2M phenotype) in the A1 domain with available 3D structures is noteworthy. The molecular effect is evident since the insertion of polar serine side chains, instead of the well-conserved aliphatic Val1409, which is buried in the protein core, is predicted to result in major disruption of the A1 domain fold or induce conformational changes in A1 that are incompatible with binding to GpIbα, thus explaining the affected patient’s phenotype. Similar

What is known about this topic?

- The gold standard for mutation detection is DNA sequencing, but for the large von Willebrand factor (VWF) gene, sequencing of the entire coding region has not yet become routine practice.
- The development of simpler, comprehensive, and precise molecular diagnostic tools that can be integrated in routine practice will offer valuable information to confirm or support the clinical and phenotypic diagnosis of von Willebrand disease (VWD).

What does this paper add?

- This paper presents a method for rapid mutation analysis by DNA sequencing of the entire VWF coding region and demonstration the utility of the method by analysis of 21 index cases representing all three VWD types.
- The method simplifies VWF detection of putative mutations and will be useful for other laboratories implicated in VWD molecular diagnostic.
- Full-length VWF gene sequencing in 21 index cases, identified a total of 30 VWF genetic variations, 12 of which are new.
phenotypes are associated with previously reported equivalent mutations (26).

Among the novel putative mutations detected in type 3 patients, the high percentage of variations potentially responsible for splice site mutations is significant. Some of these, such as 7082–2A>G (identified in 2 unrelated patients) and 7730–1G>C, have highly predicted deleterious effects, since they affect the splicing consensus sequence at the level of the nearly invariant dinucleotides at each end of the intron. The predicted impact of the potential splice site mutations described (silent nucleotide changes and intronic variations) was assessed with NetGene2 and HSF software. Molecular studies are being conducted at the mRNA level to demonstrate the true impact of these point mutations in splicing.

Two other atypical mutations were found in this study: a gene conversion that causes six consecutive nucleotide changes in exon 28, and the first VWF indel described. Gene conversion between VWF and the pseudogene has been reported as a relatively rare event that is facilitated by the high homology and presence of consensus chi or chi-like sequences in intron 27 and exon 28 of the gene (27). We describe herein the presence of a gene conversion mutation with six homozygous base changes in a family of Pakistanti origin. Surprisingly, this mutation has been reported (27) in three unrelated families from Pakistan. The discovery of this large gene conversion in four unrelated families supports the idea that the gene conversion mutation event in VWF is a fairly common occurrence (28, 29).

To our knowledge, this is the first indel mutation described as a cause of VWD (375–376delGtinsC). Indels are relatively uncommon in known gene mutations; only 1,119 indel mutations have been reported in a total of 76,011 reported mutations in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), representing 1.47% of all records.

Complete sequencing of VWF in a large number of patients and relatives, including type 2 cases, will be helpful for establishing the actual contribution of each genetic variation to the disease. We present herein an optimized procedure for sequencing essential regions of VWF that enables rapid identification of putative mutations in VWD. To our knowledge, this is the fastest and most efficient protocol to date based on complete sequencing of the VWF coding region, and combined with a recently described procedure for VWF tracking (17), it constitutes a comprehensive tool for molecular diagnosis of VWD.

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References