Rapid mutation screening in type 2A von Willebrand’s disease using universal heteroduplex generators

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Summary. Patients with type 2A von Willebrand’s disease (VWD) commonly have missense mutations in the A2 domain of the von Willebrand factor (VWF) protein. This domain is encoded by the 3' region of VWF gene exon 28 and the large majority of patients have heterozygous mutations clustered in the sequence between codons 742 and 909. We describe a DNA-based diagnostic technique which enables at least 10 previously described mutations to be rapidly identified. The method involves polymerase chain reaction (PCR) amplification of two exon 28 gene segments between codons 717–788 and 803–893, respectively. Each fragment is then hybridized with a synthetic complementary DNA molecule of similar size, termed a Universal Heteroduplex Generator (UHG). The UHG contains base deletions contiguous to the sites of known mutations and, following hybridization, allele-specific heteroduplexes are generated which can be detected by simple polyacrylamide gel electrophoresis and ethidium bromide staining. A small panel of UHG molecules covering the 3' region of exon 28 should enable the large majority of type 2A VWD patients to be rapidly diagnosed by genotype.

Keywords: von Willebrand’s disease type 2A, mutation, heteroduplex, genotype.

Approximately 15% of patients with von Willebrand’s disease (VWD) have the type 2A variant which is characterized by decreased platelet dependent function of von Willebrand factor (VWF) associated with the absence of high molecular weight VWF multimers (Ginsburg & Bowie, 1992; Sadler, 1994). Loss of the larger forms results from either impaired biosynthesis or increased degradation of the mutant protein in the plasma (Lyons et al., 1992). The large majority of type 2A mutations are found in the 3' region of exon 28 of the VWF gene which encodes the A2 domain of the VWF monomer (Ginsburg & Sadler, 1993). At least 18 amino acid substitutions have been identified which are clustered in the region of sequence between amino acid residues 742 and 909 of the VWF subunit.

Current diagnostic tests for VWD variants based on phenotype are time-consuming. Routine investigations involve measurement of the template bleeding time, plasma VIII:C, VWF:Ag and VWF:RCo, platelet aggregation responses to ristocetin and SDS–agarose electrophoresis for multimer analysis. Moreover, the results of these phenotypic assays are sometimes difficult to interpret due to physiological fluctuation. Factor VIII:C and VWF:Ag levels may also increase post-partum, following trauma and surgery and in the neonatal period. Direct detection of VWF mutations could provide a more accurate and specific approach to diagnosis. Inbal et al. (1993) have described a rapid non-radioactive method for diagnosing type 2A VWD based on hybridization between an exon 28 fragment containing the mutation and an allele-specific oligonucleotide (ASO) probe. For this approach, a panel of probes are required corresponding to each of the previously reported mutations. We have recently reported a simple and rapid technique for genotyping type 2B VWD using a method based on heteroduplex analysis (Wood et al., 1995a, 1996). The technique involves polymerase chain reaction (PCR) amplification of the gene segment containing the mutation and hybridization with a single DNA molecule of similar size termed a Universal Heteroduplex Generator (UHG). The UHG reagent is complementary to the VWF gene sequence but contains small base deletions/substitutions contiguous to the sites of known mutations. Following hybridization, allele-specific heteroduplexes are generated which can be detected by simple polyacrylamide gel electrophoresis and ethidium bromide staining.

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In this report we describe the design and construction of two UHG molecules which enable at least 10 type 2A VWF mutations to be identified. The method provides a rapid, simple and inexpensive approach to the diagnosis of this disorder by genotype and is ideally suited as a screening strategy for the routine coagulation laboratory.

MATERIALS AND METHODS

Ten patients with well-characterized type 2A VWD were studied. The VWF gene mutations in these patients were identified by direct nucleotide sequencing and are shown in Table I.

**UHG construction.** Using previously published sequences (Mancuso et al., 1991), two specific UHGs were designed to identify common VWD type 2A mutations within exon 28 of the VWF gene between codons 717 and 788 (‘upstream’ UHG) and codons 803–893 (‘downstream’ UHG) (Figs 1 and 2). The UHGs were synthesized and isolated using

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### Table I. Type 2A VWD mutations analysed by UHG technique.

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Nucleotide substitution</th>
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<tr>
<td>G742E</td>
<td>G4514A</td>
</tr>
<tr>
<td>S743L</td>
<td>C4517T</td>
</tr>
<tr>
<td>F751C</td>
<td>T4541G</td>
</tr>
<tr>
<td>L777P</td>
<td>T4619C</td>
</tr>
<tr>
<td>L817P</td>
<td>T4739C</td>
</tr>
<tr>
<td>R834W</td>
<td>C4789T</td>
</tr>
<tr>
<td>R834Q</td>
<td>G4790A</td>
</tr>
<tr>
<td>V844D</td>
<td>T4820A</td>
</tr>
<tr>
<td>S850P</td>
<td>T4817C</td>
</tr>
<tr>
<td>I865T</td>
<td>T4883C</td>
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**Fig 1.** Partial nucleotide sequence of VWF gene exon 28 (WT) and pseudogene (Pseu) between codons 717 and 788. The complementary UHG sequence is shown and contains one three-base deletion (codons 742/743) and two two-base deletions (codons 751/752 and 777). The location of four type 2A mutations (Muts) detected by the upstream UHG and the oligoprimer sequences (underlined) are also shown.© 1997 Blackwell Science Ltd, *British Journal of Haematology* 96: 464–469
previously described methods (Bidwell et al. 1994; Wood et al. 1995a). Briefly, overlapping longmers containing several identifying modifications were synthesized on an ABI 391A synthesizer using polystyrene support columns. The longmers were fused and amplified in a standard PCR reaction (see below). The amplicons were electrophoresed in a 12% polyacrylamide gel for 60 min at 200 V and visualized by ethidium bromide staining. Each full-length UHG amplicon was excised from the gel and purified by electroelution and ethanol precipitation. The precipitate was dried, redissolved, and serial dilutions were re-amplified.

**Polymerase chain reaction (PCR).** UHG and genomic DNA were amplified in 100 μl volumes using (NH₄)₂SO₄-based PCR buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, 0·01% (w/v) Tween, 1·5 mM MgCl₂, adjusted to pH 8·8 (at 25°C) containing 0·5 μM of each primer and dNTPs at a combined concentration of 200 μM. The reaction was hot-started at 72°C by the addition of 2 units of Taq polymerase (Advanced Biotechnologies Ltd, U.K.). The PCR parameters were initial denaturation at 94°C for 5 min; 32 cycles of 94°C for 30 s followed by annealing for the ‘upstream’ primers at 65°C and for the ‘downstream’ primers at 62°C for 60 s, then extension

**TYPE 2A VWD: EXON 28 DOWNSTREAM UHG**

<table>
<thead>
<tr>
<th>803</th>
<th>805</th>
<th>807</th>
<th>809</th>
<th>811</th>
<th>813</th>
<th>815</th>
<th>817</th>
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<th>821</th>
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<tbody>
<tr>
<td>WT</td>
<td>GAGGATCCGCTACAGGGCCGCCAACACACTGGGCTGCGGATCTACC</td>
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<td></td>
<td></td>
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<tr>
<td>Pseu</td>
<td>A----T- ------------ G---------T- -C-</td>
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<tr>
<td>UHG</td>
<td>A--------------C-</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Muts</td>
<td>A--------------C-</td>
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**Fig 2.** Partial nucleotide sequence of VWF gene exon 28 (WT) and pseudogene (Pseu) between codons 803 and 893. The complementary UHG sequence is shown and contains one three-base deletion (codons 834/835) and three two-base deletions (817/818, 850, 865/866). The location of six type 2A mutations (Muts) detected by the downstream UHG and the oligoprimer sequences (underlined) are also shown.

Mutation Screening in Type 2A VWD

of the primers at 72°C for 30 s and a final extension at 72°C for 10 min.

**UHG heteroduplex analysis.** 5 μl aliquots of sample and UHG PCR amplicons were electrophoresed in 2% agarose minigels in 0.5×TBE containing 0.5 μg/μl ethidium bromide, to permit visual assessment of relative amplicon concentration. UHG heteroduplex analysis was performed in a 20 μl volume, by mixing appropriate amounts of sample and UHG products to give approximately equal concentrations in the final mixture. The mixture was then denatured at 94°C for 5 min before incubating at 56°C for 10 min and a further incubation at 37°C for 10 min. DNA heteroduplexes were resolved by electrophoresis for 100 min at 200 V in a 13% non-denaturing polyacrylamide gel (2.6% cross-linking) using 1×TBE running buffer for the ‘downstream’ UHG or 120 mins at 200 V on 15% non-denaturing polyacrylamide gel (running buffer: 2×TBE) for the ‘upstream’ UHG. The specific banding patterns were visualized by ethidium bromide staining.

**RESULTS**

Using the upstream type 2A UHG covering the sequence between codons 717 and 788, heteroduplex banding patterns were generated for previously genotyped patients with mutations G742E, S743L, F751C and L777P (Fig 3). In each case, two bands corresponding to the wild-type sequence of the normal allele are seen together with one or two additional bands characteristic for each mutant allele. Rapidly migrating homoduplex fragments are seen in the lower portion of the gel. Fig 4 shows heteroduplex banding patterns for six further type 2A mutations (L817P, R834W, R834Q, V844D, S850P, I865T) in the target sequence covered by the downstream UHG between codons 803 and 893. Again, specific heteroduplex bands are obtained for each mutation. Different point mutations in the same codon (834) can be easily distinguished.

**DISCUSSION**

In this report we describe a new DNA-based diagnostic technique which enables specific gene mutations in type 2A VWD to be rapidly identified. Two UHG molecules have been constructed which are complementary to the 3’ region of the VWF gene exon 28 sequence between codons 717–788 and 803–893, respectively. The large majority of type 2A mutations are located in this region and mismatch ‘identifiers’ in the UHG reagents have been designed to detect a panel of 10 common nucleotide substitutions previously reported in the literature (Ginsburg & Sadler, 1993). The
technique is sufficiently sensitive to differentiate between two mutations situated in the same codon. Certain type 2A mutations (e.g. G742E, S743L, R834W and R834Q) occur recurrently and, based on the most recent published database, the two UHGs described in this report should provide a genotype in approximately 75% of families. However, as founder effects may be significant, the efficiency of detection will depend on the relative frequency of different mutations in the particular population or ethnic group investigated.

Direct DNA-based diagnosis may be particularly helpful when the plasma VWF level is transiently increased in stressed situations – as in the neonatal period, post-partum, and following trauma and surgery. Rapid genotype diagnosis by UHG analysis should provide a particularly cheap and effective method for performing family studies once the specific mutation has been identified in a kindred. The nature of the mutation may also influence the clinical characteristics of the disorder. For example, it has been suggested that type 2A patients with impaired secretion of high molecular weight VWF (so-called group 1 type 2A mutations) may be ‘poor responders’ to DDAVP (Englelnder et al, 1996). Furthermore, screening with both type 2A and 2B UHG reagents may also help identify and correctly classify those occasional patients who have a 2A phenotype and 2B genotype (Ribba et al, 1994; Gaucher et al, 1995).

A number of positional constraints required consideration when designing the UHG molecules used in this study. Firstly, each UHG needed to be of optimum size (150–300 base pair) and, due to the presence of the homologous VWF pseudogene, complementary to a region of wild-type exon 28 sequence which could be selectively amplified using appropriate oligonucleotides (Mancuso et al, 1991). Secondly, the UHGs are non-overlapping to eliminate the influence of two high-frequency neutral polymorphisms in exon 28 (C4641T, codon 784; A4665C, codon 792) (Sadler & Ginsburg, 1993) which would otherwise complicate the interpretation of the heteroduplex banding patterns. The technique will therefore fail to detect one previously reported type 2A mutation, L799P, which is located in the short intervening segment.

The V844D mutation was detected despite being located 18 base pairs from a two-base pair mismatch ‘identifier’ in the downstream UHG molecule. Further studies are necessary to determine whether other previously reported mutations within the sequence covered by the two UHGs but non-contiguous with the sequence mismatches can be detected. The sensitivity for identification of defects remote from a mismatch will depend on a number of factors which might be expected to influence heteroduplex conformation and electrophoretic migration. These could include the base constituents of the sequence (e.g. pyrimidine–purine ratio) the nature of the mismatches (deletion, insertion, base substitutions) and their location in the UHG molecule. However, if the current UHGs fail to detect one or more additional mutations, it is relatively simple to construct further molecules complementary to the sequence but designed with mismatches at different loci. Similarly, a small number of type 2A VWD families have been described with mutations 3' of the downstream UHG. Construction of a UHG to detect these individuals may be worthwhile if mutations in this region are found with sufficient frequency.

Using various UHG systems, we have found it uncommon for two mutations to give identical heteroduplex banding patterns. In occasional cases where patterns are similar (e.g. L817P and I865T) our strategy is to perform UHG analysis on a mixture of patient and control DNA containing the known mutation. By extending the duration of electrophoresis, small differences in heteroduplex migration are easily detected if the mutations are non-identical. Alternatively, restriction enzymes may be used to confirm the identity of the mutation if a cleavage site is eliminated or created. In the case of the L817P and I865T mutations, new sites for Msp1 and Bsr1 are generated, respectively.

The UHG technique has significant advantages compared to ASO analysis which requires synthesis of a panel of oligonucleotides corresponding to each of the mutations to be detected. No isotopic or biotin labelling of the UHG reagent is required. Furthermore, hybridization requires simple heat denaturation and cooling of the UHG-PCR product mix rather than a laborious dot-blotting procedure. Once synthesized, PCR amplification of the UHG can provide unlimited supplies of the reagent. Finally, the UHG system may, in some instances, enable previously unreported mutations to be detected if they generate novel heteroduplex banding patterns (Wood et al, 1996).

UHG-based mutation screening is ideally suited for diagnostic applications in inherited disorders which result from a small number of recurrent mutations localized to a relatively short sequence of the relevant gene. Previous applications have included rapid matching of human HLA-DW allotypes (Bidwell et al., 1993), genotype analysis in phenylketonuria (Wood et al. 1993b) and mutation screening for specific β-globin chain mutations in sickle cell anaemias and thalassaemias (Wood et al, 1993a, 1995b; Savage et al, 1995). The usefulness of the method for rapid mutation screening in type 2B VWD has also been verified recently in a series of patients who had not previously been genotyped (Wood et al, 1996). It is envisaged that a relatively small panel of UHG reagents could provide a rapid, simple and inexpensive test for genotypic diagnosis of the large majority of patients with type 2A and 2B VWD.

ACKNOWLEDGMENT

We are grateful to Professor David Ginsburg for providing DNA from VWD patients with L777P and V844D mutations.

REFERENCES


