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UHG-based Mutation Screening in Type 2B von Willebrand's Disease: Detection of a Candidate Mutation Ser547Phe

Nigel Wood[1], Graham R. Standen[2], Derrick J. Bowen[3], Anthony Cumming[4], Christopher Lush[5], Richard Lee[6], Jeffrey Bidwell[1]

From the [1]University of Bristol, Department of Transplantation Sciences, Bristol Homoeopathic Hospital Site, Cotham, Bristol, [2]Molecular Haematology Unit, Department of Haematology, Bristol Royal Infirmary, Bristol, [3]Department of Haematology, University of Wales College of Medicine, Cardiff, [4]Department of Clinical Haematology, The Royal Infirmary, Manchester, [5]Department of Haematology, Raigmore Hospital, Inverness, [6]Department of Haematology, Royal Devon and Exeter Hospital, Exeter, UK

Summary

We have recently described a novel mutation screening technique for the diagnosis of type 2B von Willebrand's disease (vWD). Analysis involves the use of a synthetic universal heteroduplex generator (UHG). To test the validity of the technique, we have applied UHG screening to seven type 2B vWD patients of previously unknown genotype. Characteristic heteroduplex patterns for Arg543Trp and Val553Met mutations were found in three patients and one patient, respectively. A fifth patient gave a novel pattern and direct sequencing revealed a hitherto unreported candidate mutation (Ser547Phe) 8 bases downstream of an "identifier" deletion in the UHG molecule. The two remaining patients gave normal heteroduplex patterns; an Arg578Gln mutation was identified by PstI digestion in one individual and no mutation could be identified in the sequence covered by the UHG in the final patient. Using a combination of UHG technology and restriction analysis, over 85% of type 2B vWD patients can be rapidly diagnosed by genotype.

Introduction

A new DNA-based diagnostic technique has been recently developed which enables specific genetic mutations in inherited diseases to be rapidly identified (1). The method involves polymerase chain reaction (PCR) amplification of the gene segment containing the mutation and hybridization with a synthetic complementary DNA molecule of similar size, termed a universal heteroduplex generator (UHG). The UHG contains base deletions/substitutions contiguous to the sites of known mutations and, following hybridization, allele-specific heteroduplexes are generated which can be detected by simple polyacrylamide gel electrophoresis. Specific mutations are thus identified by characteristic heteroduplex banding patterns. The UHG technique 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. Initial applications have included rapid matching of human HLA-DR-DW allotypes (2), genotype analysis in phenylketonuria (3) and mutation screening for specific β globin chain mutations in sickle cell anaemias (4, 5).

Correspondence to: Dr. G. R. Standen, Molecular Haematology Unit, Department of Haematology, Bristol Royal Infirmary, Bristol BS2 8HW, UK – FAX Number: +44 117 9282000

We have recently reported preliminary data on the application of UHG technology for rapid genotype diagnosis in type 2B von Willebrand's disease (6). The large majority of patients with this disorder have heterozygous point mutations in a short sequence of exon 28 of the von Willebrand factor (vWF) gene between codons 540 and 578 (7). A synthetic UHG molecule with the potential to identify seven previously reported type 2B mutations was designed and synthesized. Four common mutations gave specific heteroduplex patterns which suggested that the technique might provide a useful mutation screening procedure in this disorder (6). To test the validity of this approach, UHG screening has been performed on seven unrelated type 2B vWD patients diagnosed by conventional means who had not previously been genotyped.

Materials and Methods

UHG Construction

The design, construction and isolation of the exon 28 vWF gene UHG has been previously described in detail (1, 6). Briefly, two overlapping longmers 117 and 120 bp in size were synthesized on an AB391A synthesizer using polystyrene support columns (Fig. 1). The UHG is homologous to the true vWF gene sequence with the exception of four mismatch "identifiers": two three-base deletions in codons 544 and 551/552 and two two-base substitutions in codons 574/575 and 577/578. The longmers were fused and the 223 bp product amplified by the polymerase chain reaction (PCR) in a 100 µl reaction mixture containing 0.5 µM of appropriate oligoprimers (Fig. 1), 200 µM dNTP, 10 µl of 10 × $(NH_4)_2SO_4$-based reaction buffer and two units of thermostable DNA polymerase (Advanced Biotechnologies Ltd, UK). The PCR conditions were initial denaturation at 94° C for 5 min followed by 32 cycles of 94° C for 1 min, 55° C for 1 min, 72° C for 1 min and a final extension step of 72° C for 9 min. Following electrophoresis on a 12% polyacrylamide gel, the product was excised and isolated by electroelution and ethanol precipitation. The UHG was diluted in 75 µl of sterile water (concentration 40 ng/µl) and a 10^{-6} final dilution of the stock solution was used for routine analysis.

UHG Analysis

Leukocyte DNA was obtained from a panel of 2B vWD patients of known genotype and seven patients of previously unknown genotype. Using the above amplification conditions and oligonucleotide primers, a 229bp PCR product was isolated from each patient. An aliquot of the PCR mix was combined with an aliquot of the diluted UHG to give a final volume of 30 µl. The exact ratio for each product was defined by the intensity of the DNA bands observed following 2% agarose gel electrophoresis and the final volume ratio was adjusted to 1:1. The mixture was denatured at 94° C for 3 min before cooling to

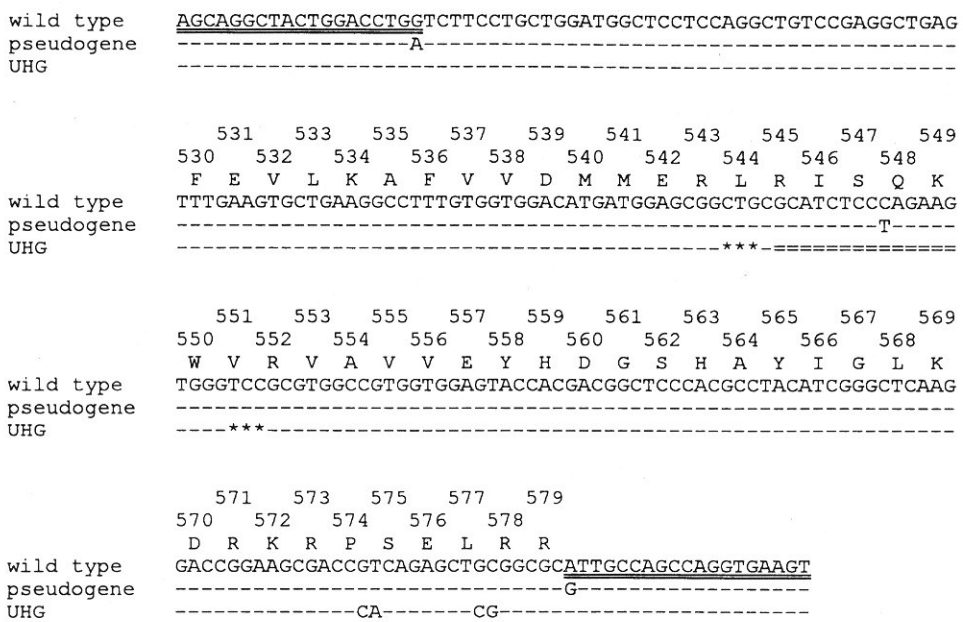

Fig. 1 Partial nucleotide sequence of vWF gene exon 28, vWF pseudogene and synthetic UHG molecule containing two three-base deletions (***) and two two-base substitutions. Oligoprimer annealing sequence (═) and a 14bp region of "longmer" overlap (= = =) are also shown

37° C at a controlled rate over 30 min. DNA heteroduplexes were resolved by electrophoresis for 90 min at 200 V on 12% non-denaturing polyacrylamide gels (2.6% crosslinking) and specific banding patterns were visualized by ethidium bromide staining.

Detection of R578Q by PstI Digestion

To identify the R578Q mutation 12 μl of the 229bp exon 28 product was digested with 1.5 μl (10 u) PstI (Boehringer Mannheim) for 3 h at 37° C. Endonucleolytic fragments were resolved by electrophoresis for 1 h at 80 V on horizontal 3.5% (w/v) fine resolution agarose gels (Metaphor, FMC Bio Products) containing 1× TBE and 0.5 μg/ml ethidium bromide.

DNA Sequencing

Nucleotide sequence analysis was performed on templates generated by asymmetric PCR (primer ratio 5:1; PCR conditions as described above) from gel purified double stranded PCR product. The products from the asymmetrical PCR were purified by gel filtration through a Sephadex G-50 spun column (1 ml) and then sequenced by the chain termination method (Sequinase™: United States Biochemical Corporation) using the nested primer:

5'GTCCTTGAGCCCGATGTAGGCGTC 3'

The products from the sequencing reactions were electrophoresed on a standard 6% polyacrylamide, 7M urea denaturing gel at 15 mA constant current.

Table 1 Coagulation parameters of seven type 2B vWD patients of previously unknown genotype

Patient	Platelet Count x 10^9/l	Bleeding Time Min	FVIII:C U/dl	vWF:Ag U/dl	vWF:RCo U/dl	Ristocetin Aggregation	Multimer Analysis
A.S.	50	>15	41	42	21	increased	HMW ↓
N.D.	20	>15	73	34	18	increased	HMW ↓
A.L.	233	>15	49	36	16	increased	HMW ↓
H.B.	214	3	44	62	43	increased	HMW ↓
J.S.	217	14	61	43	<10	increased	HMW ↓
P.B.	115	4	50	41	16	increased	HMW ↓
J.M.	135	10	41	30	25	increased	HMW ↓
NORMAL	150-400	<10	50-150	50-150	50-150		

Coagulation Assays

Plasma samples from type 2B vWD patients were assayed for FVIII:C by a one stage assay (8), vWF:Ag by either immunoelectrophoresis (9) or enzyme-linked immunoassay (10) and ristocetin-induced platelet aggregation responses and vWF:RCo by aggregometry (11). vWF multimer analysis was performed by immuno-autoradiography (12) or an immuno-linked alkaline phosphatase technique (13).

Results

The coagulation parameters of seven patients fulfilling the phenotypic criteria for type 2B vWD are shown in Table 1. In each case, an increased sensitivity to ristocetin-induced platelet aggregation (RIPA) was documented and vWF multimer analysis showed selective loss of high molecular weight (HMW) forms with normal triplet structure.

Fig. 2 shows heteroduplex banding patterns for type 2B vWD patients with exon 28 mutations previously defined by direct nucleotide sequencing. In the case of Arg543Trp, Arg545Cys, Val551Leu, Val553Met and Pro574Leu two bands corresponding to the wild type sequence of the normal allele are generated together with one or two additional bands characteristic for the mutant allele. One relatively common type 2B mutation (Arg578Gln) fails to give a pattern distinguishable from control DNA. However, the nucleotide substitution (G4022A) responsible for this mutation creates a new PstI restriction site (CTGCAG: see Fig. 1) and digestion of the amplified genomic fragment with this enzyme enables the defect to be specifically identified (Fig. 3).

Heteroduplex patterns for two patients of previously unknown genotype are also shown in Fig. 2. Patient N. D. showed a pattern characteristic for the Val553Met mutation. Patient A. S., however, gave a hitherto unrecognized pattern. The PCR fragment from this patient was directly sequenced and a C-to-T mutation at nucleotide 3929 was identified (Fig. 4). This nucleotide change corresponded to substitution of serine at position 547 by phenylalanine in the Al domain of the mature protein. The patient had typical clinical features of life-long mucosal bleeding and, in addition, fluctuating thrombocytopenia with giant platelets and spontaneous platelet aggregation in vitro. Plasma vWF multimer analysis showed a characteristic type 2B pattern (Fig. 5).

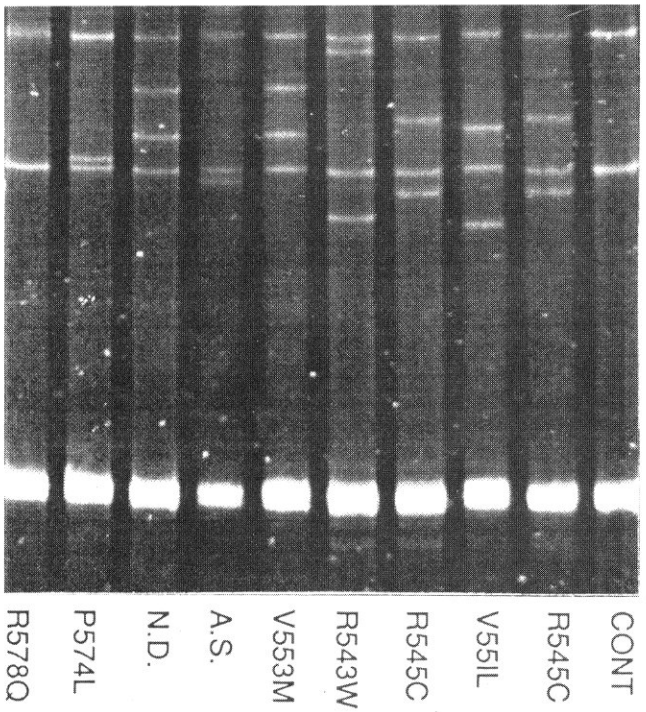

Fig. 2 Heteroduplex banding patterns for wild type gene sequence (CONT) and six common type 2B vWD point mutations. The Arg578Gln mutation gives only two bands indistinguishable from control DNA. Heteroduplex patterns for two patients of previously unknown genotype (N. D., A. S.) are also shown. Rapidly migrating homoduplex bands are seen at the bottom of the gel

UHG analysis of four other patients of unknown genotype is shown in Fig. 6. In three cases (A. L., P. B., J. M.) a heteroduplex pattern characteristic for the common Arg543Trp mutation was obtained. Patient H. B. gave a wild type pattern and an Arg578Gln mutation was confirmed by PstI restriction digestion (Fig. 3). The final patient, J. S., also gave a wild type pattern and did not have the Arg578Gln defect (data not shown). No mutation could be identified in this individual by direct sequencing the 229 bp genomic fragment.

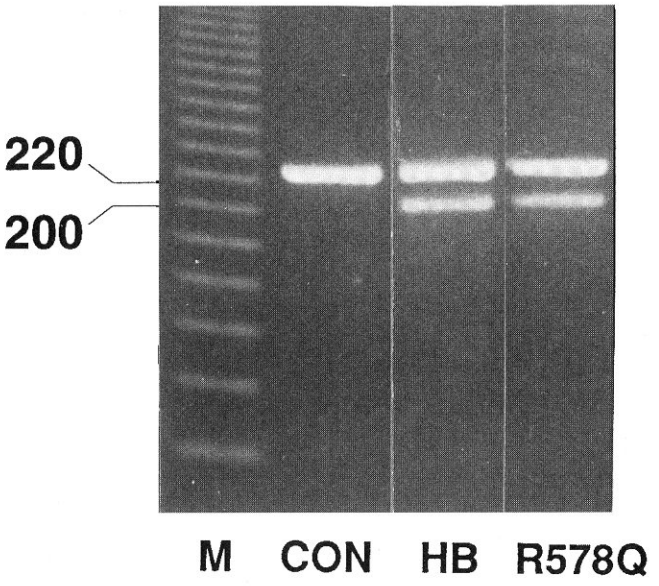

Fig. 3 Detection of Arg578Gln mutation by PstI restriction digestion of 229bp exon 28 fragment. The new restriction site produces fragments of 206 and 23 bp. M: 20 bp ladder (Advanced Biotechnologies Ltd, UK)

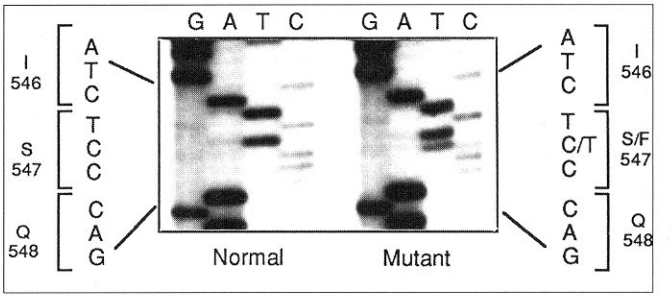

Fig. 4 Nucleotide sequence of vWF exon 28 fragment (sense strand) in patient A. S. A heterozygous C to T transition is present at position 3929 resulting in substitution of serine at codon 547 by phenylalanine

Discussion

The most recently published database of gene mutations in type 2B vWD contains 11 mutations which have been found in 44 independent families (7). Of these, 8 mutations are clustered between codons 540 and 578 and the three remaining mutations His505Asp, Leu697Val and Ala698Val have been found in occasional families only. Four mutations (Arg543Trp, Arg545Cys, Val553Met and Arg578Gln) account for approximately 90% of type 2B vWD patients studied to date.

The UHG molecule used in this study has been designed to detect all reported mutations in the sequence between codons 540 and 578.

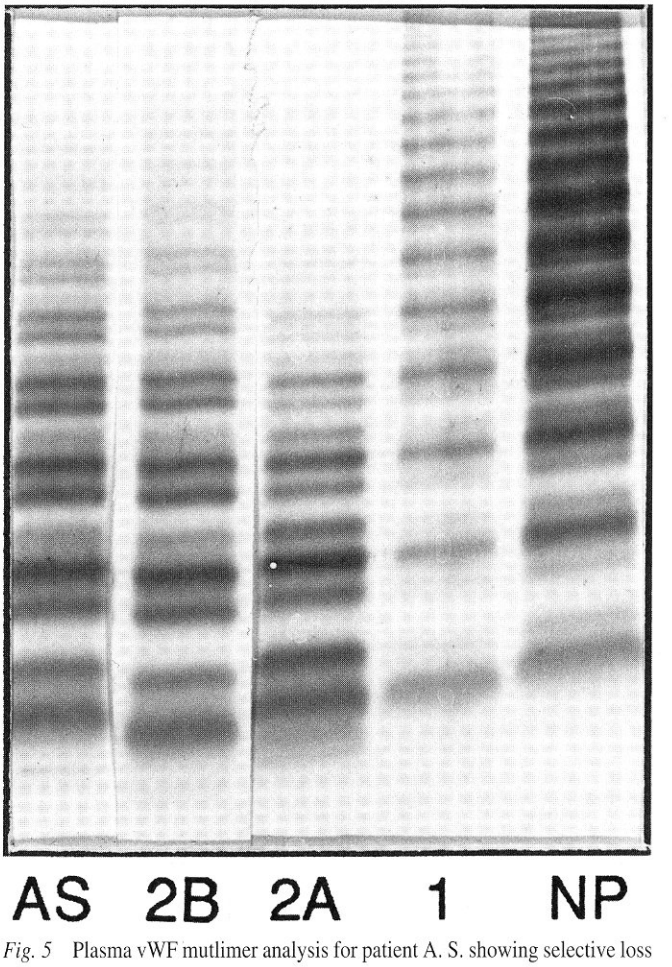

Fig. 5 Plasma vWF mutlimer analysis for patient A. S. showing selective loss of high molecular weight forms and intact triplet structure (1.2% agarose-SDS gel; immuno-alkaline phosphatase technique). Multimer patterns for normal plasma (NP), type 1, 2A and 2B vWD control samples are also shown

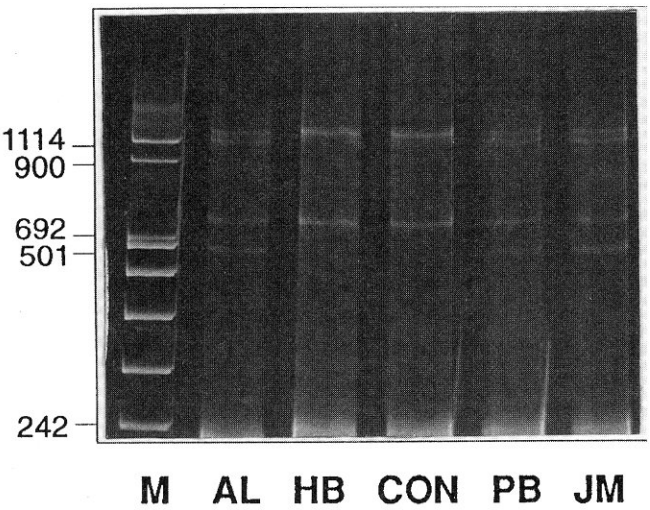

Fig. 6 UHG analysis in four unrelated type 2B vWD patients of previously unknown genotype and normal control (CON). M: molecular weight markers VIII (Boehringer Mannheim)

Analysis using previously genotyped patients confirms that specific heteroduplex patterns are seen for Arg543Trp, Arg545Cys, Val551Leu, Val553Met and Pro574Leu. The only common mutation not detected by the UHG is Arg578Gln. Most probably this is because this defect is at the 3' terminal region of the amplified genomic fragment and fails to induce significant changes in structural conformation and/or charge following interaction with the UHG molecule. As yet, we are uncertain whether minor modification of the "identifiers" in the 3' region of the UHG will enable the Arg578Gln mutation to be detected. However, since this mutation fortuitously generates a new PstI site, it can be simply and specifically identified by restriction enzyme digestion.

The present study confirms that a combination of the UHG technique and PstI restriction analysis enables nearly 90% of patients with type 2B vWD to be diagnosed rapidly and efficiently. Six out of seven patients were genotyped by this method and we believe that this diagnostic approach has important potential for application in routine coagulation laboratories. Although the UHG technique is designed to screen for specific, defined mutations, it would appear that at least a proportion of defects more remote from the UHG "identifier" mismatches may be detected. This is exemplified in the present study by the identification of a previously unreported candidate mutation (Ser547Phe) 8bp from a 3bp deletion mismatch in the UHG molecule. This defect is immediately adjacent to three previously reported type 2B mutations at codons 543, 545 and 546 within the glycoprotein 1b binding region of the mature vWF monomer (7, 14). No further examples of the Ser547Phe defect have been identified in 100 normal alleles screened by UHG analysis which suggests that the base change is not a common neutral polymorphism. However, final confirmation awaits functional studies on the recombinant mutant protein expressed in vitro.

Currently, individual UHG molecules are designed on an empirical basis (1). There is a need, however, to investigate the theoretical aspects of genomic fragment-UHG interaction and heteroduplex formation to provide a more rational basis to UHG molecule design. In particular, it is important to determine the structural factors which modify the charge and conformation of heteroduplexes and, hence, electrophoretic mobility. These might include the length of the heteroduplex, the number, type, and location of mismatches along the length of the UHG, the nature of the base pairs adjacent to the mismatch and whether the mismatch is caused by a substitution, insertion or deletion.

Although it is conceivable that two mutations could give identical heteroduplex patterns, in our experience this phenomenon has been rarely encountered. Nevertheless, it is important that the banding patterns for each of the mutations previously reported are fully defined prior to the introduction of the method for routine use.

One type 2B patient (J. S.) in our series with typical clinical features and coagulation parameters gave a wild type heteroduplex pattern on UHG screening and PstI digestion excluded the common Arg578Gln mutation. A hitherto undescribed mutation within the sequence covered by the UHG but outside the influence of the "identifier" mismatches could conceivably explain the observation but was excluded by direct nucleotide sequencing. A small number of mutations in exon 28 of the vWF gene immediately upstream and downstream of the UHG target sequence have been identified in occasional type 2B families (7, 15, 16) and patient J. S. may fall into this category. If further examples of such mutations are identified and are relatively localized, synthesis of additional UHG molecules to cover the target sequence might be appropriate.

An eight-month-old daughter of the patient with the novel Ser547Phe mutation had previously been noted to have thrombocytopenia and spontaneous platelet aggregation in vitro. UHG heteroduplex analysis was performed on DNA derived from a heel prick blood sample dried on filter paper and inheritance of the mutant type 2B allele confirmed (data not shown). We have previously suggested that UHG analysis should provide the means for rapid neonatal diagnosis in infants at risk of inheriting this disorder (6).

Our findings in type 2B von Willebrand's disease support the view that UHG-based analysis has important potential for rapid genotype diagnosis in many monogenic inherited disorders. Diseases characterized by frequent mutations localized to relatively short segments of nucleotide sequence are particularly amenable to this approach. In combination with automated gel reading technology, the technique can provide a useful tool for mutation detection in population screening programmes.

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