Mutational analysis of the von Willebrand factor gene in type 1 von Willebrand disease using conformation sensitive gel electrophoresis: a comparison of fluorescent and manual techniques

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ABSTRACT

Two versions of conformation sensitive gel electrophoresis, fluorescent (F-CSGE) and manual (M-CSGE) techniques, were compared for mutation analysis of the von Willebrand factor gene. 56 PCRs were used to amplify all 52 exons of the gene in seven type 1 von Willebrand disease cases, plus a healthy control. One hundred and ninety-two samples were analyzed on each F-CSGE gel, compared with 40 on M-CSGE. 125 amplicons revealed bandshifts using F-CSGE, but only 101 by M-CSGE. Five mutations were detected by both techniques. F-CSGE detected 45 different polymorphisms whereas M-CSGE detected only 39. F-CSGE is high-throughput and more sensitive than M-CSGE.

Key words: CSGE, F-CSGE, mutation analysis, von Willebrand's factor, von Willebrand's disease.

Type 1 von Willebrand disease (VWD), a common autosomally inherited bleeding disorder, results from partial quantitative deficiency of plasma von Willebrand factor (*VWF*), and is the most common form of VWD accounting for 60%-80% of all cases.[1,2] The VWF gene (*VWF*) is large and has proved challenging to screen for mutations. It covers 178kb, has 52 exons and is located at chromosome 12p13.3. A partial pseudogene, *VWFP* corresponding to exons 23-34 is present on chromosome 22q11.22-23[3,4] and complicates *VWF* analysis. Over 130 DNA polymorphisms have been reported in the coding and closely flanking intronic regions of VWF as detailed on the international VWF database (*http://www.vwf.group.shef.ac.uk/*).

Identification of disease-causing mutations in large genes such as VWF remains challenging in terms of speed, accuracy and cost-effectiveness. The gold standard for mutation detection is DNA sequencing, but for the large *VWF* gene, sequencing the entire coding region has not yet become routine. Several mutation screening techniques such as denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism analysis (SSCP) and conformation sensitive gel electrophoresis (CSGE) can be used. Sensitivity estimates for detection of sequence variation using these methods range from 35% to 100%, depending on the size and sequence context of the DNA examined.[5-7] Comparison of manual CSGE (M-CSGE), fluorescent CSGE (F-CSGE) and direct DNA sequencing for exon 11 of the BRCA2 gene in 16 individuals showed concordance of the results obtained by the three methods and established the high sensitivity of the F-CSGE.[8] In the present study, we have utilized CSGE on a fluorescent platform to attempt to increase its throughput and sensitivity for mutation detection for patients enrolled in the *Molecular and clinical markers for the diagnosis and management of type 1 VWD* study (MCMDM-1VWD).[9-11]

Design and Methods

Study population

Index cases (IC) of seven European families who all had a personal and family history of type 1 VWD were analyzed. One healthy control (HC) was also included. All individuals were enrolled in the EU FP5 MCMDM-1VWD study.[11]

DNA extraction and amplification

DNA was extracted from citrate anticoagulated venous blood according to standard procedures. PCR amplification from genomic DNA was conducted on a Perkin-Elmer 480 thermocycler in a 50 μL volume. 52 exons and intron/exon boundaries plus the 3' region of VWF were amplified in a mixture containing 1.1x Readymix PCR Master Mix (ABgene, Epsom, UK) with 1.5, 2.5 or 4.0 mmol/L MgCl2 and 50 nM of the appropriate primers as previously reported[12-14] and listed on *http://www.euvwd.group.shef.ac.uk/*. Samples were initially denatured at 97°C for 5min, DNA amplification was achieved by 35 cycles of denaturation (95°C, 60 sec), annealing for 60 sec and extension (72°C, 90 sec).

Manual conformation sensitive gel electrophoresis (M-CSGE)

The method of Williams et al was used.[15] Briefly, 5 μL of PCR product from each IC was mixed with an equal volume of PCR product from the HC. Samples were denatured at 98°C for 5min and then incubated at 65°C for 30 min to allow heteroduplex formation. For comparison, HC amplicons were self-heteroduplexed and 2-5 μL of heteroduplexed samples were loaded onto a 10% CSGE gel.

Fluorescent conformation sensitive gel electrophoresis (F-CSGE)

The method of Ganguly *et al*. was used.[8] Briefly, each forward primer was labelled with FAM, HEX or TET fluorescent dye and on the basis of size/dye, the amplicons were divided into 19 groups of three (eg group 8; exons 7, 17 and 22) to enable multi-loading of products onto each gel lane. Heteroduplexes prepared as above were diluted from 1 to 15-fold (dependant on PCR product concentration) in deionized water followed by mixing 1 μL of each diluted heteroduplex with the two other heteroduplexes in the same group. Sample loading mix contained 1.5 μL of the three mixed PCR products with 0.5 μL size standard GenoTYPE™ ROX 50-500 (Applied Biosystems), 0.5 μL dextran blue (50 mg/mL) and 0.5 μLfreshly deionized formamide in a total volume of 3 μL. Of this mixture, 1.5 μL was loaded onto a 10% polyacrylamide gel consisting of 99:1 acrylamide:BAP (Fluka), 15% deionized formamide in 1xTBE. The gel was prepared in a final volume of 5 0ml and polymerized overnight with 0.6% (w/v) ammoni-um persulphate and 0.06% (v/v) TEMED. F-CSGE gels were 420 mm×250 mm in size and 0.2 mm thick. Sixty-four sharkstooth well combs were used to form wells. Electrophoresis was performed under mildly denaturing conditions at 2000Volts for 4.5 hours at 30°C in 1xTBE with a 36cm well-to-read distance. Fragment size analysis software (Genescan and Genotyper, Applied Biosystems) was used for data analysis.

DNA sequencing

PCR products with aberrant CSGE patterns were analyzed using automated DNA sequencing (Applied Biosystems PRISM 377) according to the manufacturer's instructions.

Results and Discussion

The coding region of the VWF gene of seven index cases from the MCMDM-1VWD study plus a control were screened for mutations using both fluorescent and manual CSGE. In total, 448 amplicons (8×56) were screened using both M- and F-CSGE. A hundred and one amplicons showed migration shifts using both techniques, while 24 amplicons in which shifts were unclear (smeared or very faint), or undetected by M-CSGE, were seen as migration shifts by F-CSGE. Migration shifts were seen in an average of 16 amplicons per individual using F-CSGE (range 12-19), but only 13 amplicons per individual using M-CSGE (range 10-16). Five candidate mutations were detected by both techniques, and are listed in Figure 1 (parts A to D).

F-CSGE detected shifts more efficiently than M-CSGE (Figure 1). For example, migration shifts detected using F-CSGE, were not detected (E) or were ambiguous (F) using M-CSGE. DNA sequencing confirmed the changes: a known single nucleotide polymorphism (SNP), c.4141G→A, in (E) and two different known SNPs in (F); heterozygous c.4641C→T plus homozygous c.4801C→A. DNA sequencing of amplicons demonstrating F-CSGE migration shifts revealed 49 different SNPs including eight novel changes, while 39 of the same SNPs were identified using M-CSGE. The eight novel changes were each located in different VWF introns. Apart from a false-positive result for exon 5 using both techniques, a low false-positive rate was seen only with F-CSGE. DNA sequencing did not reveal any nucleotide change in 4% (5/125) of amplicons demonstrating a F-CSGE migration shift.

In order to screen VWF for mutations in type 1 VWD, it was necessary to analyze at least 18.5 kb of DNA sequence per individual. It has been previously shown that mutation pre-screening followed by DNA sequencing for mutation identification in large genes is cost-effective and efficient.[16] Using CSGE pre-screening in this study, and sequencing only 3 to 5kb of selected amplicons (10-15 amplicons/individual of 56 in total),
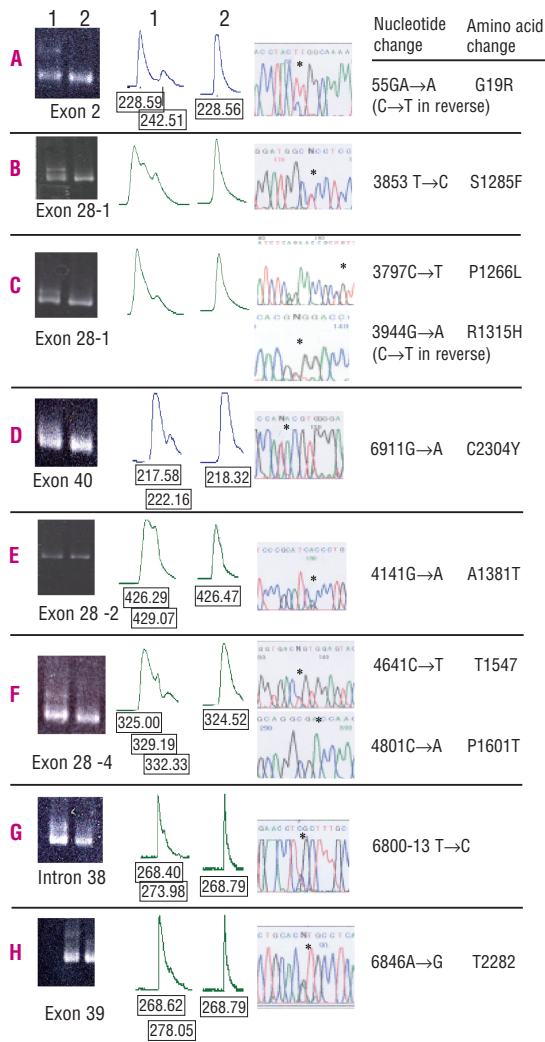
Figure 1. Examples of M- and F-CSGE. In each panel A-H, the M-CSGE image in lane 1 shows the aberrant bands in the index case (if any) and lane 2 shows the pattern from the normal control. F-CSGE trace 1 shows the result obtained from the index case and trace 2 shows the result from a normal control. Traces obtained using Genotyper software for F-CSGE analysis show the peak size underneath each trace corresponding to the heteroduplex on the gel. Nucleotide and amino acid changes identified by DNA sequencing are also shown in each part. *indicates the position of a nucleotide change. A to D: Five candidate missense mutations identified in the VWF gene in this study are indicated. F-CSGE peak sizes in B and C are not shown because the amplicon size of 515bp was larger than the size standard (50 to 500bp). Nucleotide changes resulting in migration shifts are shown in A (exon 2, c.55G→A; p.G19R), B (exon 28, c.3944T→C; p.S1285F), C (exon 28, c.3797C→T; p.P1266L plus c.3853G→A; p.R1315H on the same allele) and D (exon 40, c.6911G→A; p.C2304Y). E. Amplicon 28-2 did not show a bandshift in M-CSGE (lanes 1 and 2), while F-CSGE demonstrated aberrant bands in comparison with the normal pattern (trace 1 and 2), resulting from exon 28, c.4141G→A; p.A1381T. F. Aberrant band in exon 28-4 was unclear using M-CSGE (lane 1), while two migration shifts were seen using F-CSGE (trace 1). DNA sequencing results revealed two different previously reported polymorphisms, heterozygous c.4641C→T; p.T1547 and homozygous c.4801C→A; p.P1610T. G and H. Examples of the sensitivity of F-CSGE. Two different single-base substitutions in a 267-bp PCR product covering intron 38 and exon 39 revealed two different patterns with different peak sizes. DNA sequencing demonstrated two different nucleotide changes, a novel intronic change c.6800-13T→C and a known polymorphic change c.6846A→G; p.T2282.

delivered a high throughput method in terms of both time and cost saving in comparison with direct DNA sequencing alone. The multiload option was selected as a simple way to optimize F-CSGE gel use. PCR products were divided into groups of three amplicons and multi-loaded onto each gel lane. A hundred and ninety-two samples were analyzed on each 64 well gel, whereas only 40 samples were analysed on each M-CSGE gel.

The sensitivity of any mutation scanning method is determined by its ability to detect and distinguish between different single-base substitutions. The length of the M-CSGE gel was 41cm and sample migration was 25 to 35cm after 17 hours (depending on amplicon size) when the gel was documented. In F-CSGE, all amplicons migrated the full length of the gel (36cm) and were detected at its base. The increased migration length leads to enhanced resolution. Figure 1 (G and H) demonstrates the ability of F-CSGE to detect different nucleotide substitutions in the same amplicon, through different peak sizes. F-CSGE detected insertions and deletions as well as single base substitutions in this study, as previously reported.[8,17,18]

Ten amplicons gave shifts identified as sequence alterations for some or all individuals using F- but not M-CSGE (those for exons 4, 14, 18, 19, 24, 28-2, 28-3, 28-4, 35 and 39). Most discrepancies were amplicon- and not patient- specific. Some, such as those for amplicons 28-4 and 35 were subtle changes that were missed by M-CSGE due to a minor migration change, but were identifiable using F-CSGE due to automated size-calling of the shifted peak. Analysis of the size and GC content of these ten amplicons did not show any common feature to be responsible for lack of sensitivity of M-CSGE (size 217-478bp, GC content 47-65%).

A false-positive result for exon 5 was demonstrated for both techniques. Migration shifts in all samples were not associated with sequence alteration. A small number of other false-positive results were seen in F-CSGE, but no common features of amplicons giving rise to F-CSGE shifts were identified. False-negative results are difficult to quantitate, but overall in the MCMDM-1VWD study, candidate mutations were found in 105 out of 150 IC (70%) using techniques including CSGE, SSCP, DHPLC and DNA sequencing.[11] This is similar to the detection rate in this small sub-group. Significant improvements from modifying the methodology to the fluorescent platform resulted in a higher throughput, reproducible and more rapid method than M-CSGE, with enhanced resolving power for seeking sequence alteration in the large *VWF* gene. The utility of F-CSGE has been recognized by its adaptation for use on capillary sequencers as fluorescent conformation sensitive capillary electrophoresis.[19,20]

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