Analysis of the \textit{F8} Gene in Individuals with High Plasma Factor VIII:C Levels and Associated Venous Thrombosis

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Summary

High FVIII:C levels have previously been shown to be an independent risk factor for thrombosis with 4.8 times higher potential risk of thrombosis in individuals with FVIII:C levels greater than 1.5 u/ml. Recently, we found that raised FVIII:C levels are largely attributable to elevated FVIII:Ag levels. The determinants of FVIII:Ag levels are unclear and might be partly genetic. The promoter of the \textit{F8} gene has recently been characterised and consequently we investigated the promoter and the 3’ terminus of the \textit{F8} gene for possible polymorphisms associated with raised FVIII:Ag levels in 62 selected individuals with a thrombotic tendency. We confirm previous reports that raised FVIII:C levels are largely attributable to an elevation in FVIII:Ag and this is also associated with elevation of vWF; non-O blood group; relatively short APTT and relatively low APC ratio. We screened 1140bp of the proximal promoter including the protein binding sites identified by DNase I footprint analysis by SSCP, however no polymorphisms were identified. Direct DNA sequence analysis of the region -542 to +165 failed to identify any sequence polymorphisms. The recently described polymorphism in the polyadenylation cleavage site in the prothrombin gene associated with increased prothrombin activity prompted us to screen the region surrounding the 3’ terminus of the \textit{F8} gene for polymorphisms but we found none.

Introduction

The pathogenesis of thrombosis is complex. Episodes of venous thrombosis in patients with a demonstrable inherent predisposition to thrombosis are usually precipitated by acquired prothrombotic risk factors. It has been suggested that inherited predisposition to thrombosis in an individual usually results from combinations of mutations in two or more genes that encode proteins involved in coagulation (1, 2).

Recent evidence has suggested that high levels of FVIII coagulant activity (FVIII:C) are associated with a significantly increased risk of venous thrombosis. The Leiden Thrombophilia Survey found that increased FVIII:C was an independent risk factor for thrombosis, however, it was suggested that raised FVIII:C is not in itself sufficient to cause thrombosis but in combination with other factors, high FVIII:C levels present a strong thrombosis risk. The estimated relative risk for thrombosis in patients with FVIII:C levels greater than 1.5 u/ml was 4.8 (3). We recently demonstrated that the elevated FVIII:C in patients with thrombosis is largely attributable to elevated levels of FVIII:Ag, due either to increased synthesis or enhanced stability of FVIII in plasma (4). We excluded common causes of acquired increased circulating levels of FVIII such as malignancy and acute phase response. In the absence of evidence for an acquired stimulus to over production of FVIII this suggests a genetic basis for these high levels.

In recent years a steadily increasing number of reports have documented the importance of polymorphisms in directly modulating transcription of genes encoding not only proteins important in haemostasis but in other physiological processes. For example, polymorphic variation in the promoter region of the protein C gene has been shown to influence transcriptional efficiency \textit{in vitro} and to be correlated with plasma protein C levels (5, 6). A sequence length polymorphism (4G/5G) in the plasminogen activator 1 (PAI-1) gene promoter region, 675 base pairs upstream of the transcriptional start site, also correlates significantly with levels of PAI-1 in plasma (7). A G/A polymorphism at -455 in the 5’ flanking region of the β-fibrinogen gene has been found to be correlated with plasma fibrinogen levels (8). Similarly, a 10 base pair insertion at -323 in the factor VII promoter region correlates with FVII plasma levels (9). More recently, a G to A substitution in the polyadenylation site of the prothrombin gene has been shown to be associated with an increase in prothrombin activity and increased thrombotic risk (10).

In this study we have selected a group of individuals with increased FVIII:C plasma levels and analysed both the sequences flanking the start of transcription and the sequences flanking the polyadenylation signal of the \textit{F8} gene for DNA polymorphisms that may be linked to or responsible for the raised plasma concentrations of FVIII.

Materials and Methods

Subjects and Blood Samples

Individuals were recruited from the Thrombosis Clinic at Hammersmith Hospital. Blood samples of three more patients suffering from thrombembolic disease, persistently raised FVIII levels and family history of venous thrombosis were referred with requests for further analysis. Sixty two individuals fulfilled the selective criteria of a raised FVIII:C level (>1.5 u/ml). Fifty six were patients who suffered one or more incidents of venous thrombosis. Six more individuals had a strong family history of venous thrombosis.

Blood samples for the study were drawn at least 3 months after the last thrombotic event and only two of the patients were on oral anticoagulant therapy at the time of study. None of the female subjects were pregnant or using oral contraceptives at the time of study. Other hereditary anticoagulant deficiencies or acquired disorders associated with thrombosis, for example, lupus anticoagulant, malignancies were excluded. Six of the selected individuals proved to be FV Leiden positive by genetic testing.

Citrated, and if possible, additional EDTA and clotted blood specimens were obtained from the antecubital vein of each patient after informed consent.
Haematological and Coagulation Tests

Activated thromboplastin time (APTT) was performed according to the standard method immediately after separation of the plasma. The reagent used was Diagen kaolin/platelet substitute from Diagnostic Reagents Ltd, Oxon, England.

FVIII:C assays were done by the one-stage clotting method, using FVIII deficient substrate (Immuno, Vienna, Austria): Kaolin/platelet substitute mix - ture was the same as used for the APTT. All coagulation assays were performed on an ACL-300R analyser (Instrumentation Laboratories Ltd, Warrington, England) and referenced to a FVIII:C standard supplied by Immuno (Vienna, Austria) which is calibrated against the international standard for FVIII-related activities (NIBSC, South Mimms, UK).

FVIII:Ag levels were determined by a sandwich type ELISA technique: dilutions of citrated test plasma were incubated on welled plates coated with a mouse capture monoclonal antibody (12A4, donated by Professor IR Peake, Sheffield) followed by addition of horseradish peroxidase labelled anti-FVIII antibody, that was extracted and purified from the plasma of a haemophilia A patient who developed a FVIII inhibitor with a titre of 8 400 NIH units/ml (11). FVIII:Ag levels were referenced to a FVIII:Ag standard supplied by Stago (Stago Asserachrom FVIII Ag; Shield Diagnostics, Dundee, UK) which is calibrated against the international standard for FVIII-related activities (NIBSC, South Mimms, UK).

von Willebrands factor Ag was assayed by an ELISA technique, using rabbit anti-human vWF antibodies from Dako (High Wycombe, England).

Activated protein C (APC) resistance test was performed according to the clotting method of Coatest (Chromogenix, Mölndal, Sweden) based on the original method described by Dahlbäck (2).

Citrated plasmas were separated within two hours of collection. Assays on citrated specimens were either performed immediately after separation or aliquotted and stored at –20°C until further testing. EDTA and citrated packed cells specimens were kept frozen at –80°C until DNA extraction.

Fig. 1 Sequence of the promoter region of the human F8 gene (accession number M88628). Showing the protein binding sites identified by Figueiredo & Brownlee (21) in grey shading. The oligonucleotides (EM1-5) used in the study are underlined and overscored with an arrowhead indicating the 3’ end. The restriction endonuclease sites for the enzymes used in the SSCP analysis are shaded in dark grey, ▶ indicates the transcription start site. The 5’ flanking sequence is shown in lower case and exon 1 sequence in uppercase. The start of coding sequence is indicated by the translated sequence. The nucleotides are numbered from the start of transcription (+1).

Fig. 2 Sequence of the 3’ terminus of the human F8 gene. The sequence of exon 26 is shown in bold uppercase and the 3’ flanking sequence in lowercase. The oligonucleotides used for the PCR amplification and direct sequencing (EM6 and 7) are shaded in dark grey and overscored with an arrowhead indicating the 3’ end. The 3 polyadenylation consensus sequences are shaded in light grey. The nucleotides are numbered according to the complete sequence of the exon 26 F8 gene fragment (accession number M88648)
Anticoagulant screening included ATIII, protein C (functional), protein S (total and free) was carried out by standard methods.
All the above tests were performed on citrated plasma.
ABO blood grouping was done by routine methods.
Erythrocyte sedimentation rate (ESR) or plasma viscosity was carried out using a Coulter plasma viscometer.
Complete blood count was obtained by flow cytometry on an automated cell analyser (Sysmex).
C-reactive protein (CRP) was determined by the Bayer CRP assay, an immunoturbidimetric assay run on a Bayer AXON analyser (ref SM-40183D910, reference kit no T01-1940-01, calibrated using Bayer set point calibrants and two SPS calibrants from the protein reference unit, Sheffield, England).
Above tests were performed on EDTA anticoagulated blood samples and CRP on serum.

**DNA Analysis**

DNA was extracted from EDTA anticoagulated whole blood or from the packed cells of citrated blood specimens. Extraction was performed according to the method of Kunkel (12) or using a commercial DNA extraction kit according to the manufacturer’s instructions (Nucleon Biosciences, Scotlab, England).

The factor V Leiden polymorphism was detected by the PCR site-directed mutagenesis method of Rabès et al. (13).

The 1142 base pairs of 5’ sequences spanning the transcription start site of the F8 gene were analysed by SSCP and direct DNA sequence analysis. SSCP analysis was performed as described by Michaelides et al. (14). PCR products were labelled by the incorporation of α-33P d-ATP (111 kBq/100 µl reaction, 337-110 TBg/ml; Amersham Life Science, Buckinghamshire, England). Primers EM1 and EM2 (Fig. 1) were used for the PCR amplification. Thermal cycling conditions were: 7 min 94°C, 30 cycles of 94°C 1 min, 58°C 1 min, final extension 72°C 10 min. The PCR products were digested with the restriction enzymes Ava II and EcoRI (Promega, Madison, Wisconsin, USA) prior to SSCP analysis.

Consecutive SSCP was performed on the 464bp product obtained following EcoRI digestion of the PCR product amplified using a biotinylated primer EM2 (Fig. 1) and 33P labelling (14). The biotinylated 464bp product following EcoRI digestion was captured by strepavidin-coated magnetic beads (Dynabeads® M-280 Streapavidin, Dynal AS, Oslo, Norway) and further digested with Mse III (Boehringer, Mannheim GmbH, West-Germany) prior to SSCP analysis.

PCR products were directly sequenced using the ABI PRISM™ Dye Terminator Sequencing Ready Reaction kit and AmpliTaq® DNA Polymerase, FS according to the manufacturer’s instructions (Perkin-Elmer Applied Biosystems, Warrington, Great Britain) and analysed on an ABI Applied Biosystems 373A DNA automated sequencer (Perkin-Elmer Applied Biosystems, Warrington, Great Britain). The sequencing primers EM2-5 (Fig. 1) were used to analyse the 706 base pairs immediately spanning the transcription start site of the F8 gene.

The 296 base pairs of sequence spanning the polyadenylation site of the F8 gene were PCR amplified using the oligonucleotides EM6 and EM7 (Fig. 2). Thermal cycling conditions were: 7 min 94°C; 30 cycles of 93°C 1 min, 58°C 1 min, 72°C 5 min; final extension 72°C 10 min.

**Results**

**Subjects**

Sixty two individuals who fulfilled the selective criteria of a raised FVIII:C level (>1.5 µ/ml) were included in the study. Twenty one (33%) were male, 41 (66%) were female. Fifty two (84%) were of Western European descent, 8 (13%) were of Asian origin and 2 (3%) were of African origin. Their ages ranged from 16 to 66 years, mean and median ages 40.7 years and 37 years, respectively. More female than male patients at presentation fell into a younger age group: of the 56 patients who suffered thrombosis, 21 of 35 females (60%) were younger than 40 years compared to 8 of 20 males (40%). Clinical diagnosis of the patients included: deep vein thrombosis which was the most frequent diagnosis: 26 patients (46.4%), followed by pulmonary embolism: 15 patients (27%); 4 patients (7%) had both and 10 patients (18%) had suffered more than one thrombotic event. Six of the individuals had never suffered from thrombosis but had a strong family history of thrombosis. The remainder of the patients had thrombosis at less common sites: cerebral 6 (10.7%), retinal 2 (3.5%), jugular 1 (1.7%), inferior vena cava 1 (1.7%). Two of the females were on oral contraceptives when thrombosis occurred.

**Coagulation and Haematological Analysis**

The results of the coagulation and haematological tests are shown in Table 1. In addition the ABO blood grouping was determined. A: 22 (45%); B: 19 (39%); AB: 1 (2%); O: 7 (14%); ratio non-O:O = 6:1 (n = 49).

Statistical analysis using Spearman Ranking Correlation Coefficient revealed that increased FVIII:C correlated positively with increased FVIII:Ag and with increased vWF: correlation coefficients were r = 0.3, p = 0.05 and r = 0.25, p = 0.07, respectively. A positive but not significant correlation between FVIII:Ag and vWF was found (r = 0.11, p = 0.76).

**FV Leiden Mutation Analysis**

Five of the individuals were heterozygous for the FV Leiden mutation (RS06Q): 4 females and one male. Two of these individuals had a family history only of venous thrombosis but no personal history. One Middle Eastern male patient was found to be homozygous for FV Leiden and had his first thrombotic event at the age of 47 years. The APC ratios of all these individuals were at the lower cut off levels of normal or decreased (range 1.45-2.1; mean 1.88). The FVIII:C levels of these 6 individuals were scattered within the range of the studied patients. Mean FVIII:C levels were 2.02 u/ml and 2.27 u/ml for subjects with the FV Leiden mutation and the remaining subjects studied, respectively.

**Table 1. Coagulation and haematological data**

<table>
<thead>
<tr>
<th>Test (normal range)</th>
<th>No analysed</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII:C (0.45-1.5 µ/ml)</td>
<td>62</td>
<td>2.25</td>
<td>1.5 - 4.1</td>
</tr>
<tr>
<td>FVIII:Ag (0.45-1.5 µ/ml)</td>
<td>53</td>
<td>1.73</td>
<td>0.62 - 4.01</td>
</tr>
<tr>
<td>vWF (0.45-1.5 µ/ml)</td>
<td>53</td>
<td>1.27</td>
<td>0.41 - 2.95</td>
</tr>
<tr>
<td>APTT (30-46 sec)</td>
<td>59</td>
<td>32.5</td>
<td>25 - 42</td>
</tr>
<tr>
<td>APCR 2.0-4.0 males; 2.0-3.5 females</td>
<td>58</td>
<td>2.44</td>
<td>1.45 - 3.74</td>
</tr>
<tr>
<td>Fibrinogen (1.5-4.0 mg/ml)</td>
<td>55</td>
<td>2.8</td>
<td>1.7 - 4.7</td>
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<tr>
<td>ESR (0-15 mm/hr Westergen)</td>
<td>51</td>
<td>17.9</td>
<td>1 - 141</td>
</tr>
<tr>
<td>CRP (&lt;10 µg/ml)</td>
<td>56</td>
<td>6.18</td>
<td>1 - 43</td>
</tr>
<tr>
<td>WBC (4.0-11.0 x 10^3/L)</td>
<td>56</td>
<td>7.33</td>
<td>3.5 - 13.6</td>
</tr>
</tbody>
</table>
SSCP Analysis of the F8 Gene Promoter

A 1182 base pair fragment corresponding to –997 to +185 (Fig. 1) was PCR amplified from DNA extracted from each subject. The 32P-labelled PCR product was digested with the restriction enzymes EcoR I and Ava II prior to SSCP analysis yielding fragments of 327, 120, 271 and 464bp. The 464bp EcoR I fragment corresponding to –279 to +185 base pairs was further screened by consecutive SSCP following digestion with the restriction endonuclease Mae III generating fragments of 191 and 273bp. No difference in mobility of any fragment analysed was observed between the patients and normal subjects analysed in parallel.

DNA Sequence Analysis of the Promoter and 3′ Terminus of the F8 Gene

The region –542 to +165 base pairs of each patient was analysed further by direct DNA sequencing of the PCR amplified products. Direct DNA sequence analysis was also performed on PCR amplification products spanning the polyadenylation site of the FVIII mRNA (Fig. 2).

No nucleotide substitution, polymorphism or mutation, could be detected in these two regions in any of the 62 studied individuals.

Discussion

In this study we have analysed a group of patients with thrombosis and elevated levels of FVIII:C, plasma levels. We confirm previous reports that the rise is largely attributable to an elevation in FVIII:Ag (r = 0.3; p = 0.05); and is also associated with elevation of vWF (r = 0.25; p = 0.07); non-O blood group: (non-O:O = 6:1) (3, 4); relatively short APTT (mean = 32.5 sec) (15); relatively low APC ratio, excluding the FV Leiden positive individuals (mean = 2.52). It is known that an increased FVIII:C can cause a decrease in the APC ratio but individuals with FV Leiden do not necessarily have increased FVIII:C (16). High FVIII:C in the presence of FV Leiden can act as an additive risk factor for the development of thrombosis (17). It has been found that about 25% of patients with previous thrombosis, decreased APC ratios but without FV Leiden, have functional APC resistance associated with FVIII levels in excess of 1.5 u/ml (18). Six individuals in the present study (9.7%) had the FV mutation (a prevalence slightly lower than is usually found for this mutation among patients with venous thrombosis. However, not all the individuals in this study had suffered thrombosis). The reduction in APC ratios provides a possible mechanistic link between thrombosis and elevation of FVIII:C, but the purpose of this study was to determine whether a genetic basis for FVIII:C elevation could be found.

Other factors known to affect levels of FVIII:C include increasing age, pregnancy, use of oral contraceptives and hormone replacement therapy, infectious or inflammatory disease and malignancy. Since FVIII is an acute phase reactant (19) we have used ESR, CRP and fibrinogen levels as markers of the acute phase response. We excluded patients with major inflammatory responses but it is possible that part of the genetic effect is an exaggerated response to acute phase mediators and these patients are equally valid subjects for study. Three individuals in this study could qualify for having raised FVIII:C as part of an acute phase reaction but this would not explain their preceding thrombotic event(s) (4). Malignant disease was excluded clinically. It was also found that patients on oral anticoagulant therapy have raised FVIII:C up to 8 weeks after withdrawal of the drug (20). In our study 36 of 56 patients had more than one FVIII:C determination and in only five did the FVIII:C level tend to decrease with time. It could be that some of our patients demonstrated a transient rise of FVIII:C levels after oral anticoagulant withdrawal. The minimum time of FVIII determination in our study was 3 months after the thrombotic event and after oral anticoagulant withdrawal.

The promoter of the human F8 gene has recently been characterised (21, 22). DNase I footprint analysis showed the presence of 19 protein binding sites distributed along the region –1005 to +164 of the F8 promoter (Fig. 1). Functional analysis revealed that the region from –109 to +107 contains all the necessary elements for maximal promoter activity. We have screened 1140bp of the proximal promoter including the binding sites A to S identified by DNase I footprint analysis by SSCP. No fragments with aberrant mobility were identified. The fragments analysed by SSCP were at the upper limit for efficient mutation identification therefore we sequenced the F8 promoter region from –542 to +165 in all 62 individuals with raised FVIII:C levels and either a personal history or a strong family history of thrombosis, however, no mutation or polymorphism could be identified.

The recently described G to A substitution in the polyadenylation cleavage site in the prothrombin gene associated with increased prothrombin activity and increased thrombotic risk (10), prompted us to investigate the 3′ region of F8 gene. The region flanking the 3′ end of the F8 gene containing 3 AATAAA consensus polyadenylation signals (Fig. 2) was PCR amplified and directly sequenced for each individual, however, no nucleotide substitutions could be identified.

The failure to identify a mutation/polymorphism in the promoter and 3′ terminus does not preclude a role for F8 gene sequences in determining levels of FVIII:Ag. The recent study by Kamphuisen et al (23) suggests that an X-linked locus is likely to have some effect in determining FVIII:C levels. This locus may of course lie anywhere on the X chromosome but the F8 gene remains the most likely candidate. Additional regulatory sequences may lie further 5′, within the body or 3′ of the gene.

In conclusion, high FVIII:C has previously been shown to be an independent risk factor for thrombosis with 4.8 times higher potential risk of thrombosis in individuals with FVIII:C levels greater than 1.5 u/ml (3). We have confirmed that the raised FVIII:C levels are largely attributable to elevated FVIII:Ag levels. We have investigated the promoter and the 3′ terminus of the F8 gene for possible polymorphisms associated with raised FVIII:Ag levels in 62 selected individuals with a thrombotic tendency but found none.

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References


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