

MUTATIONS AT THE ACTIVATED PROTEIN C CLEAVAGE SITES ARG336 AND ARG562 OF FACTOR VIII IN THAI PATIENTS WITH VENOUS THROMBOSIS

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Abstract. Venous thrombosis is a multicausal disease, more than one genetic risk factor may cooperate to effect thrombotic risk. Factor V Leiden is found to be an important hereditary risk factor for venous thromboembolism. Analogous to factor V Leiden, a point mutation at amino acid positions Arg336 and Arg562 in factor VIII may predispose patients to thrombosis. Eighty-one Thai patients with venous thrombosis and 100 Thai healthy volunteers have been studied. Neither heterozygous nor homozygous mutations were detected both thrombosis patients or normal volunteers. However, further studies with larger samples of venous thrombosis patients are recommended.

INTRODUCTION

Thromboembolic diseases remain a major cause of morbidity and mortality in most countries. Factor V Leiden (FVL) has been known to be an important hereditary risk factor for venous thromboembolism (Rosendaal, 1999). FVL is a point mutation in the factor V gene (guanine to adenine replacement at nucleotide position 1691), causing the substitution of arginine 506 by glutamine at the cleavage site of activated protein C (APC) (Bertina *et al.*, 1994). FVL is found predominantly in Caucasian populations, varying from 4 to 15%, depending on geographic location, but it is rare in Asia (Rees *et al.*, 1995).

The discovery of FVL was a major stimulus for laboratories around the world to search for other genetic polymorphisms and mutations predisposing individuals to thromboembolic events, such as prothrombin G20210A,

methylene tetrahydrofolate reductase (MTHFR) C677T and recently, the two mutations involving Arg306 at the APC cleavage site of factor V (Poort *et al.*, 1996; Arruda *et al.*, 1997; Chan *et al.*, 1998; Williamson *et al.*, 1998; Bertina, 1999).

We have investigated the FVL, Arg306 and Arg679 of the factor V gene and the G20210A of the prothrombin gene in Thai thrombotic patients and in the Thai population. We found that FVL and factor V Hong Kong (Arg306Gly) were detected 11-14% and 4.3% in Thai patients with venous thrombosis (Arnutti *et al.*, 1998; Hiyoshi *et al.*, 1999; Prayoonwiwat *et al.*, 2000). Factor V is cleaved by APC at residues Arg306 and Arg506 that are homologous to residues Arg336 and Arg562 in factor VIII (Roelse *et al.*, 1996). Analogous to FVL, a mutation at amino acid position Arg336 and Arg562 of factor VIII may predispose patients to thrombosis. Because the function of factor VIII in the coagulation system is very homologous to factor V, it is possible that any mutation on these cleavage sites can potentially affect the APC inactivation process and the APC resistant (APC-R) factor VIII may lead to hereditary thrombophilia due to its APC-R phenotype.

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In this study, we have further investigated the mutation of factor VIII. The Arg336 and Arg562 cleavage sites in factor VIII for APC inactivation were studied to ascertain whether mutations at these sites occur or have any association with patients suffering from venous thrombosis.

MATERIALS AND METHODS

Subjects

Eighty-one patients with apparent venous thrombosis, who attended the Division of Hematology, Department of Medicine, Phramongkutklo Hospital, Bangkok, Thailand were enrolled in this study. Their ages ranged from 19 to 72; the mean age was 44 years. There were 41 males and 40 females. A control group of 100 unrelated healthy volunteers without any thrombosis were included in this study from the Blood Bank, Army Institute of Pathology, Bangkok, Thailand. Their ages ranged from 23 to 52; the mean age was 36 years. There were 74 males and 26 females. Informed consent was obtained from all subjects.

Extraction of DNA

Blood was collected in EDTA anticoagulant and genomic DNA was prepared by phenol-chloroform method or using a commercial DNA extraction kit according to the manufacturer instructions (Pel-Freeze, WI, USA).

Polymerase chain reaction (PCR)

Analysis of the factor VIII mutation at the

Arg336 and Arg562 of factor VIII was carried out using the PCR for the detection of the CGA-codon of Arg336 and the AGA-codon of Arg562 by a slight modification of the originally described method (Roelse *et al.*, 1996). The amplification solution contained 100-300 ng DNA, 100 pmol of each primer, as shown in Table 1, 200-400 µmol of each dedoxynucleoside triphosphate, which were mixed in a total volume of 50 µl of 1x PCR reaction buffer with 1.25 units of Amplitaq Gold DNA polymerase (Perkin Elmer, New Jersey, USA). The reaction mixture was placed in a PTC 200 Thermal Cycler (MJ Research, Inc, MA, USA) and for the detection of the CGA-codon of Arg336, the mixture was performed at 95°C for 2 minutes, 20 minutes at 50°C, 3 minutes at 72°C and subjected to 50 cycles of amplification (95°C for 45 seconds, 50°C for 90 seconds, and 72°C for 3 minutes) with a final incubation at 65°C for 5 minutes. PCR for the detection of the AGA-codon of Arg562 was performed at 95°C for 5 minutes, 20 minutes at 55°C, 3 minutes at 72°C and subjected to 50 cycles of amplification (95°C for 45 seconds, 55°C for 90 seconds, and 72°C for 3 minutes) with a final incubation at 65°C for 5 minutes.

Restriction digestion

Fifteen microliters of PCR product for the detection of the CGA-codon of Arg336 was digested overnight with either *RsaI* (New England Biolabs, MA, USA) or *TaqI* (New England Biolabs, MA, USA) and analyzed by agarose gel electrophoresis. The PCR product

Table 1
Sequences of oligonucleotide primers used in this study.

Primer	Nucleotide sequence	Position
F8-336-1	5'-AGCTGTCCAGAGGAACCCCAACTATC-3'	nt 1039-1063
F8-336-3	5'-AGTTTTAGGATGCTTCTTGGC-3'	nt 1180-1200
F83-336-2	5'-AGCTGTCCAGAGGAACCCCAAGTA-3'	nt 1039-1062
F8-562-1	5'-TGCTACAAAGAATCTGTAGATCGA-3'	nt 1717-1740
F8-562-2	5'-CTAAGCTCATTCCTTACTGACC-3'	nt 87-108 intron 11

Underline: mismatch oligonucleotide

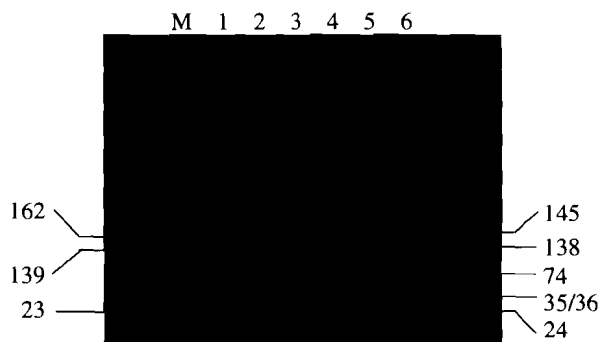


Fig 1—PCR-RFLP assay for factor VIII at the Arg336 and Arg562. Lane 1, 3: 162 bp of non-digested PCR product for FVIII at the Arg336. Lane 2,4: Fifteen microliters of PCR product were treated with *RsaI* and *TaqI*. Normal alleles resulted in 139, 23 bp and 138, 24 bp, respectively. Lane 5: 145 bp of non-digested PCR product for FVIII at the Arg562. Lane 6: 74, 35 and 36 bp of PCR product of normal alleles after being treated with *MboII*. Lane M: The EZ Load 20 bp molecular ruler was used to estimate the size of the fragments obtained.

for the detection of the CGA-codon of Arg562 was digested with *MboII* (New England Biolabs, MA, USA).

RESULTS

Eighty-one patients with venous thrombosis and 100 healthy volunteers have been studied. Any mutation in the CGA-codon of Arg336 abolished both *RsaI* and *TaqI* restriction sites. Normal and substituted alleles were distinguished by the sizes of the obtained restriction fragments: 162 bp for any mutation of Arg336; 139 and 23 bp for normal alleles digested with *RsaI* and 138 and 24 bp for normal alleles digested with *TaqI*. Normal and mutation alleles were distinguished by the sizes of the restriction fragments: 74, 35 and 36 bp for normal alleles; 145 bp for any mutation in the AGA-codon of Arg562 (Fig 1).

Neither heterozygous nor homozygous mutations, at the activated protein C cleavage

sites in Arg336 or Arg562 of factor VIII were detected in both Thai thrombosis patients and normal Thai volunteers. Interestingly, two DNA samples from the venous thrombotic patients could not be identified following normal procedures, even when the amplified product was treated with *TaqI* enzyme as described by manufacturer instructions. Only when the *TaqI* enzyme was increased ten-fold could a clear result be obtained. This result was also confirmed by DNA sequencing.

DISCUSSION

Venous thrombosis is a multicausal disease with resulting more than one genetic risk factor which may co-operate to effect thrombotic risk (Bovill *et al*, 1999). In the thromboembolic Thai patients, the heterozygous alleles of FVL and factor V Hong Kong (Arg306Gly) were found (Arnutti *et al*, 1998; Hiyoshi *et al*, 1999; Prayoonwiwat *et al*, 2000). However, the cause of thrombosis in some cases could not be explained. Additional gene point mutation studies need to be performed in order to explain the cause of thrombosis. Although this study has not identified any mutation at the Arg336 and Arg562 of factor VIII, it supports the theory that mutations at the activated protein C cleavage sites at Arg336 and Arg562 of factor VIII may not play a major role in pathogenesis of venous thrombosis (Roelse *et al*, 1996; Hooper *et al*, 1998).

Although, DNA sequencing is a gold standard for detecting gene point mutations, it is costly and time-consuming. Especially in the laboratory with a limited budget, an automatic sequencer may be unavailable. In this study, the PCR-RFLP (restriction fragment length polymorphism) method was used because it can be performed using only electrophoresis, a restriction enzyme, and a PCR machine, without an expensive automatic sequencer. In addition, this method is suitable for dealing with a large number of specimens at the same time, which might be useful for further investigation.

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REFERENCES

- Arnutti P, Hiyoshi M, Prayoonwiwat W, *et al.* Coagulation factor V Leiden mutation was detected in the patients with activated protein C resistance in Thailand. *Thromb Haemost* 1998; 80: 344-5.
- Arruda VR, von Zuben PM, Chiaparini LC, Annichino-Bizzacchi JM, Costa FF. The mutation Ala667 → Val in the methylenetetrahydrofolate reductase gene: a risk factor for arterial disease and venous thrombosis. *Thromb Haemost* 1997; 77: 818-21.
- Bertina RM, Koeleman RPC, Koster T, *et al.* Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; 369: 64-7.
- Bertina RM. Molecular risk factors for thrombosis. *Thromb Haemost* 1999; 82: 601-9.
- Bovill EG, Hasstedt SJ, Leppert MF, Long GL. Hereditary thrombophilia as a model for multigenic disease. *Thromb Haemost* 1999; 82: 662-6.
- Chan WP, Lee CK, Lam CK, Liang R. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. *Blood* 1998; 91: 1135-9.
- Hiyoshi M, Hashimoto S, Tagawa S, Arnutti P, Prayoonwiwat W, Tatsumi N. A Thai patient with the mutation of Arg306 of FV gene identical to the Hong Kong but not to the Cambridge type. *Thromb Haemost* 1999; 82: 1553-4.
- Hooper WC, Dilley H, Austin H, *et al.* Absence of mutations at APC cleavage sites Arg306 in factor V and Arg336, Arg562 in factor VIII in African-Americans. *Thromb Haemost* 1998; 79: 236.
- Prayoonwiwat W, Arnutti P, Hiyoshi M, *et al.* Detection of factor V Leiden in Thai patients with venous thrombosis. *Asian Pac J Allergy Immunol* 2000; 18: 105-8.
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; 88: 3698-703.
- Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995; 346: 1133-4.
- Roelse JC, Koopman MMW, Buller HR, *et al.* Absence of mutations at the activated protein C cleavage sites of factor VIII in 125 patients with venous thrombosis. *Br J Hematol* 1996; 92: 740-3.
- Rosendaal FR. Risk factors for venous thrombotic disease. *Thromb Haemost* 1999; 82: 610-9.
- Williamson D, Brown K, Luddington R, Baglin C, Baglin T. Factor V Cambridge: A new mutation (Arg306 → Thr) associated with resistance to activated protein C. *Blood* 1998; 92: 1119-24.