Phenotypic and genetic analysis of dysprothrombinemia due to a novel homozygous mutation

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ABSTRACT

Objective: We study the phenotype and genotype of a novel gene mutation of factor II (FII) that leads to dysprothrombinemia, and do the meta-analysis to illuminate its molecular pathogenesis. It will further contribute to our comprehension of the pathogenesis of this type of disease.

Methods: The prothrombin time (PT), activated partial thromboplastin time (APTT) and the activities of other factors were determined by the one-stage clotting method. The prothrombin antigen was measured with enzyme-linked immunosorbent assay (ELISA). Function of the mutant protein was evaluated by thrombin generation tests. Potential mutations in exons, exon–intron boundaries and 5',3' untranslated sequences of prothrombin gene were screened by polymerase chain reaction and direct sequencing. Suspected mutations were confirmed by reverse sequencing. The structure change of this protein was analyzed by model and bioinformatics analyses.

Results: Phenotypic analysis revealed that the proband had an obviously prolonged PT, APTT, reduced prothrombin activity but normal antigen levels. The other tests were normal. Sequencing analysis detected a homozygous g.26329T>G in the catalytic domain resulting in p.Tyr510Asp. His parents and uncle were heterozygous for this mutation. The thrombin generation test showed that the mutant protein had obstacles in thrombin generation. Bioinformatics and model analyses illuminated that the mutation will be probably damaging and perturbing the structure of Na+-binding site, which will affect the activation of prothrombin.

Conclusion: This was the first report of such a mutation in the position which was associated with dysprothrombinemia.

KEYWORDS
Prothrombin; dysprothrombinemia; mutation analysis; prothrombin deficiency

Introduction

The prothrombin (factor II, FII) is a vitamin K-dependent zymogen, which was synthesized primarily in the liver, and composed of 579 amino acid residues. The FII molecule contains a γ-carboxyglutamic acid domain (Gla, 1–40), two kringle domains (41–271) and a catalytic serine protease domain (271–579) [1]. It is encoded by the gene F2, which was located on chromosome 11p11-q12, including 14 exons and 13 introns. The thrombin contains 308 amino acid residues, which is generated from prothrombin under the splitting at Arg271-Thr272 by activated factor X (FXa) [2]. The main role of prothrombin was to convert fibrinogen (FIB) into fibrin under the catalysis of the prothrombinase complex (activated factor X-activated factor V-calcium ion-Platelet phospholipids, FXa-FVa-Ca²⁺-PL). Also, it participates in anticoagulation process via activating protein C system. In addition, it plays roles in inflammatory, cell proliferation and tissue repair processes, too [3].

Hereditary prothrombin deficiency is one of the rare congenital coagulation defects with a prevalence of 1 in 2 000 000, which happened 10-fold more in the regions where consanguineous marriages are customary [3,4]. The different plasma contents of functional and antigenic levels of prothrombin lead to two types: hypoprothrombinaemia, which is characterized by a concomitant decrease in prothrombin antigen and activity, and dysprothrombinemia, with the feature of decreased activity but borderline-normal or normal antigen. The severity of bleeding manifestations is generally related to the plasma prothrombin level. But, the absolutely complete prothrombin deficiency has not been diagnosed so far, suggesting that this condition is incompatible with life [4]. In this paper, we described a patient who was homozygous for a novel mutation resulting in the dysprothrombinemia.

Materials and method

Patients

The proband was a 21-year-old man, who was admitted into our hospital due to blurred vision of his left eye for a month, after a series of inspection, he was diagnosed as ‘conjunctival hemorrhage’. The routine coagulation tests showed prolonged prothrombin time (PT), and activated partial thromboplastin time (APTT) (34.3s and 112.9s, reference range: 13.1–14.1s and 31.5–40.5s), but normal thrombin time (TT) and fibrinogen
His factor VII activity (FVII:C), FX activity (FX:C), FV activity (FV:C) was normal, while the prothrombin activity (FII:C) was prominently reduced to 3% (reference range: 86–108%), the prothrombin antigen (FII:Ag) was normal (102%, reference range: 80–120%). The patient said he had suffered from easy subdermal ecchymosis and gingival bleeding since childhood. There was no evidence showed liver or kidney dysfunction and bleeding tendency. There were a total of five members of two generations of the family participated in our study (Figure 1). His parents are consanguineous without clinical history of bleeding.

**Laboratory index tests**

The Ethics Committee of the First Hospital Affiliated to Wenzhou Medical University (China) approved all our studies. The blood samples were collected from the patient and his family members into the sodium citrate anticoagulant tubes for coagulation tests. PT, APTT, TT, FIB, FVII:C, FX:C, FV:C and FII:C were measured on the Stago STA analyzer (Diagnostica Stago, Asnière sur Seine, France) with one-stage clotting method. And enzyme-linked immunosorbent assay (ELISA) kit (Changfeng, Wenzhou, China) were used to measure the FII:Ag and the level of the thrombin-antithrombin complex (TAT).

**Thrombin generation analysis**

To evaluate the whole influences of the mutations on blood coagulation, the thrombin generation tests were did according to the method CAT described by Hemker et al. [5]. All operations were following the manufacturer’s protocol. The detector was Fluoroskan Ascent FL reading meter (Thermo Eletron and Fisher Scientific company, U.S.A.). The reagents (5 pmol/l tissue factor, 4 µmol/l phospholipid and fluorogenic substrate) and the analysis software were supported by Thrombinoscope BV company (Netherlands). The reactions were monitored for 60 minutes. Four parameters (lag time, peak height, time to peak (Ttpeak), endogenous thrombin potential (ETP)) were used to assess thrombin generation dynamics. We also measured the thrombin generation assay in the absence and presence of thrombomodulin (TM) to detect the ability of the residual activity of mutated thrombin to activate protein C (PC).

**DNA analysis**

Genomic DNA of the enrolled members was extracted from peripheral blood leucocytes using established commercialized kit (Tiangen, Beijing, China). Twelve pairs of primers, covering all the exons and their flanking regions of the prothrombin gene, were designed according to the gene sequence from the PubMed (GenBank M17262), the primer sequences were listed in (Table 1). All the PCR amplifications were performed in 25 µl reaction system containing 12.5 µl PCR amplification reaction regent (2×) (Tiangen, Beijing, China), 8 µl water, 1 µl of each primer (10 µM), 2 µl genomic DNA (100 ng). The DNA samples were denatured at 95°C for 5 minutes, then 95°C for 30 seconds, respective melting temperatures for 30 seconds and 72°C for 30 seconds followed by 30 cycles, at last extended at 72°C for 10 minutes on the Applied Biosystem Thermal Cycler 2720 (ABI, Foster City, CA, USA). Putting 5 µl PCR amplified products in 1.5% agarose gel to detect the amplification effect through electrophoresis. After purification, PCR products were directly sequenced at Sunsoon BIO-Technology Corporation (Shanghai, China). Regarding the genomic GenBank (M17262) as reference, the results were analyzed by BLAST program. Hundred individuals were selected to exclude the polymorphism.

**Bioinformatics analysis**

The possibility that each newly identified missense mutation could be pathogenic was assessed using the bioinformatics tool: Polymorphism Phenotyping-2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/index.shtml). To analyze the molecular structure of
the mutant protein, the protein model was generated by spdbv software and PIC programs (Protein Interactions Calculator, http://pic.mbu.iisc.ernet.in) based on the three-dimensional structure in the Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do, PDB ID: 4NZQ).

Results

Coagulation tests

The phenotypes of all the family members studied are shown in (Table 2). The proband presented with reduced FII:C of 3% but normal FII:Ag. The level of his TAT was normal. Concordantly reduced activity and normal antigen were observed in his father, mother and uncle.

Thrombin generation analysis

Comparing with the patients’ parents, the proband had more obstacles in thrombin generation with the prolonged ratio of Lag time (3.39%), Tpeak (2.32%) and significantly reduced ratio of ETP (12.6%), Peak height (3.9%) (Figure 2). In the absence or presence of TM, the ability of the residual activity of mutated thrombin to activate PC was same as the normal control. Hence, the fact that the mutant protein affects plasma thrombin generation may be closely correlated with the coagulation tests in our proband.

DNA analysis

Sequence analysis of F2 gene revealed the proband carried a homozygous g.26329T>G point mutation in exon 13, leading to the substitution of Tyr (TAC) for Asp (GAC) at the site of 510 in the catalytic serine protease domain, which was according to the Tyr239 of the thrombin. The enrolled family members, except for his aunt, were all heterozygous for the missense mutation. In a control group consisting of 100 individuals, the mutation (p.Tyr510Asp) was absent.

Bioinformatics analysis

The score of PolyPhen-2 was 0.995 which revealed the substitution was probably damaging for the prothrombin protein. Model analyzing demonstrated Tyr510 interacts with Ile321, Leu485, Pro486 through hydrophobic bonds, and with Asn484 through main chain-side chain hydrogen bonds in the wild type. The replacement of Tyr510 with Asp510 resulted in a main chain-side chain hydrogen bond between Asp510 and Lys516, as well as the disappearing of all the hydrogen bonds (Figure 3).

| Region | Primer sequence (5′→3′) | Amplified fragment length (bp) |
|--------|--------------------------|-------------------------------|
| 5′UTR  | F: GAACCTAGATATTCATGG    | 600                           |
|        | R: TGCTCTGCGCCAGAGG      |                               |
|        | 1                   |                               |
|        | F: TAAGACGCATATACATGGCTC |                               |
|        | R: AAGGACGCACTCTCCTTCAA  | 449                           |
|        | 2                   |                               |
|        | F: GACTGCCGTGTCTCTGAGGTC |                               |
|        | R: TGAATGAGCCTGTAGAGCAG  | 331                           |
|        | 3                   |                               |
|        | F: CGGTGTGFGTGGGAGGAACTCC|                               |
|        | R: CAATTGCAAGTCTGGGGGACT | 640                           |
|        | 4                   |                               |
|        | F: CAACTTCCAGGGAGAGAG    |                               |
|        | R: TGCTCCGGTACCCAGATATTTC| 504                           |
|        | 7                   |                               |
|        | F: CTGAGCTACACAGGACAG    |                               |
|        | R: CCGCTGTGCTATCCCCACT   | 488                           |
|        | 8                   |                               |
|        | F: AGGTATTTGCGCAGTACGCC  |                               |
|        | R: ATGTCTGACTGTGTCACAT   | 601                           |
|        | 10                  |                               |
|        | F: AATAAAAGTGAGGGCAGAGG |                               |
|        | R: GCTGGGATGTATATTTCTGG  | 490                           |
|        | 11                  |                               |
|        | F: GACACATCTGTCACAGAAGAC|                               |
|        | R: AGTGGGAACCTCCAGTAAAA  | 462                           |
|        | 12                  |                               |
|        | F: GTCTCTAGAAGTGGCGTTG   |                               |
|        | R: ACTCTATTAACCTCCCCCAT  | 444                           |
|        | 13+14               |                               |
|        | F: ATGTGATGAGGGAAGGATG  |                               |
|        | R: GTCTTTTTATGAGGAAACCA | 585                           |
|        | 3′UTR               |                               |
|        | F: TCTGAAGACAGTGCCTGGCC |                               |
|        | R: TCCAGTAGTATTACGCGTC  | 392                           |

The main phenotypic detection results of the pedigree of family members.

| PT (s) | APTT (s) | TT (s) | FIB (g/l) | FII:C (%) | FVII:C (%) | FX:C (%) | FII:Ag (%) |
|--------|----------|--------|----------|-----------|-----------|----------|------------|
| Proband| 34.3     | 112.9  | 15.9     | 2.78      | 98        | 91       | 94         | 3          | 102        |
| Father | 13.2     | 32.7   | 16.2     | 3.33      | 102       | 105      | 112        | 52         | 106        |
| Mother | 14.0     | 40.0   | 17.5     | 3.20      | 99        | 89       | 101        | 55         | 99         |
| Uncle  | 13.5     | 33.7   | 18.2     | 2.42      | 108       | 114      | 108        | 54         | 111        |
| Aunt   | 13.9     | 35.4   | 17.9     | 2.43      | 104       | 111      | 113        | 91         | 110        |
| Reference range | 11.7–14.8 | 29.0–43.0 | 14.0–20.0 | 2.00–4.00 | 86–110 | 84–120 | 95–113 | 86–108 | 80–120 |
Discussion

The gene sequencing of patients with prothrombin deficiency always fell into two categories: (1) compound heterozygote for two different mutations; (2) homozygote for a single mutation. In our study, the proband was category 2, presented with a novel homozygous mutation p.Tyr510Asp in the exon 13. His parents who share a blood line were all heterozygous for the mutation. The phenomenon like these is very rare in China, because it was prohibited by legislation. The fact prothrombin antigen was normal and activity was obviously decreased indicated that the synthesis and secretion of mutated protein was normal but its functional was impaired. As far as we are concerned, this is the first report of p.Tyr510Asp which is associated with dysprothrombinemia.

In our study, conservative analysis revealed that Tyr510 is fully conserved within macaque, chimpanzee, sheep, cow, chook and pig while the case is not the same in the vitamin K dependent protein (data not shown). During the progress of evolution, the Tyr510 residue maintains conserved, which suggested its importance in maintaining the normal structure and function of the protein. It was found that the Na⁺ binding loop of the specific carboxyl terminal region

![Figure 2](image_url). The results of thrombin generation tests.

![Figure 3](image_url). Model of the missense mutation. (a) Wild type. The lines in blue and green represent hydrophobic bonds and hydrogen bonds, respectively. (b) Mutant type. The line in yellow represents ionic bond.
of prothrombin was composed of 8 highly conserved amino acid residues located 20 Å away from the thrombin catalytic triad (His363-Asp419-Ser525) [6]. And vitro studies have shown that substitution of amino acids on the Na+ binding loop can alter the Na+ binding environment, thereby disrupting the balance between thrombin coagulation and anticoagulant activity [1,7].

Tyr510 is located in the catalytic domain of the heavy chain, next to the catalytic triad and Na+ binding loop, which can ensure its high activity toward physiological substrates [8]. Model analysis (Figure 3) revealed that the replacement of uncharged Tyr510 by a negatively charged Asp510 would disrupt the original hydrophobic bonds between Tyr510-Ile321, Tyr510-Leu485, Tyr510-Pro486 and the hydrogen bonds between Tyr510-Asn484, but generated another hydrogen bonds between Asp510-Arg517 and an ionic bond between Asp510-Lys516. These changes in chemical bonds could affect the normal space structure and function of the FII protein. There was a study demonstrated that the Arg517 took part in the formation of the ionic cluster, which is helpful for Na+ positioning correctly [1]. Hence, we can safely speculated that the mutation may affect the activation of prothrombin and perturb the structure of Na+ binding site, resulting in decreased prothrombin activity.

Up to now, approximately 60 variants associated with prothrombin deficiency were listed in the Hmuan Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/all.php), including 46 missense mutations, three splicing mutations, four regulatory mutations and seven frameshift mutations. Looking up literatures and databases, there were 22 mutations associated with dysprothrombinemia were found (Table 3), of which most were missense ones, and they are located most on the exon 8, 9, 10, 11, 12, 13, 14, the clinical features of which vary from asymptomatic to severe. The pathogenic mechanism of these mutations leading to dysprothrombinemia was either destroyed the conversion progress of prothrombin to thrombin or disturbed the ability of thrombin curdled fibrinogen. The identification of abundant mutations is important for us to study the relationship between structure and function of the protein. Herein, we identified a novel missense mutation in the exon 13 leading to dysprothrombinemia with mild clinical features. The performance of asymptomatic maybe explained by the statement that both procoagulant and anticoagulant functions of Tyr510 are impaired.

In our case, the thrombin generation test result of the proband showed that the procoagulation function of prothrombin was damaged, but, as the content of TAT and the ability of mutated thrombin to activate PC were normal, so we could draw the conclusion that the mutation only affected the procoagulation function without the anticoagulant function. Further investigations are under way to elucidate the precise pathogenic mechanism.

To conclude, we have found a novel homozygous mutation (p.Tyr510Asp) in the exon 13 of prothrombin gene in a Chinese proband. The mutation is responsible for the prothrombin deficiency reported here. Identification of the prothrombin deficiency in this family allowed mutation-specific prenatal screening.

| Table 3. Phenotype and genotype characteristics of a family with dysprothrombinemia. |
|-----------------------------------------------|-----------------|-----------------|------|-------|-----------------|------------------|
| Mutation      | Location | Domain         | Type           | Genotype | FII:C | FII:Ag | Clinical feature | Reference       |
| Arg271Cys     | E8       | Activation     | Missense       | Hom     | <10   | >100  | Severe          | [9,10]          |
| Arg271His     | E8       | Cleavage site  | Missense       | Het     | 52    | 100   | Mild            | [11]            |
| Glu300Lys     | E9       | Catalytic      | Missense       | Hom     | 0.82  | Normal | Severe          | [16]            |
| Gly319Arg     | E9       | Catalytic      | Missense       | Hom     | 7     | Normal | Severe          | [14]            |
| Arg320His     | E9       | Activation     | Missense       | Het     | 44    | 115   | Severe          | [15]            |
| Val322Glu     | E9       | Catalytic      | Missense       | Hom     | 5     | 40    | Mild            | [4]             |
| Gly330Ser     | E9       | Catalytic      | Missense       | Hom     | 10    | Normal | Asymptomatic    | [17]            |
| Met337Thr     | E10      | Catalytic      | Missense       | Hom     | 1     | 61    | Asymptomatic    | [4]             |
| Arg382His     | E10      | Catalytic      | Missense       | Hom     | 2     | 25    | Asymptomatic    | [12]            |
| Arg382Cys     | E10      | Catalytic      | Missense       | Hom     | 10    | 50    | Mild            | [18]            |
| Gly341stop    | E14      | nonse          |                  |         |       |       |                 |                 |
| Arg418Trp     | E11      | Catalytic      | Missense       | Hom     | 13    | 28    | Severe          | [19]            |
| Arg457Gln     | E12      | Catalytic      | Missense       | Hom     | 18    | 100   | Asymptomatic    | [20]            |
| Glu466Ala     | E12      | Catalytic      | Missense       | Comp het | <1   | 31    | Mild            | [21]            |
| Gly476stop    | E12      | Catalytic      | Missense       | Comp het | 51   | 102   | Asymptomatic    | [1]             |
| Arg517Gln     | E13      | Catalytic      | Missense       | Hom     | 2     | 70    | Asymptomatic    | [22]            |
| Gly458Ala     | E14      | Catalytic      | Missense       | Hom     | <3     | 60    | Asymptomatic    | [23]            |
| Asp552Glu     | E14      | Catalytic      | Missense       | Hom     |         |       |                 |                 |
| Lys556Thr     | E14      | Catalytic      | Missense       | Hom     | 58    | 110   | Asymptomatic    | [7]             |
| Gly559Val     | E14      | Catalytic      | Missense Not shown |         | <2%   | Normal | Asymptomatic    | [24]            |
**Ethics approval**

Our study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (China).

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**References**

[1] Henriksen RA, Dunham CK, Miller LD, et al. Prothrombin Greenville, Arg517→Gln, identified in an individual heterozygous for dysprothrombinemia. *Blood*. 1998;91(6):2026–2031.

[2] Adams TE, Huntington JA. Structural transitions during prothrombin activation: on the importance of fragment 2. *J Biochimie*. 2016;122:235–242.

[3] Lancellotti S, De Cristofaro R. Congenital prothrombin deficiency. *Semin Thromb Hemost*. 2009;35(4):367–381.

[4] Akhavan S, Mannucci PM, Lak M, et al. Identification and three-dimensional structural analysis of nine novel mutations in patients with prothrombin deficiency. *Thromb Haemost*. 2000;84(6):989–997.

[5] Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003;33(1):4–15.

[6] Di CE, Guinto ER, Vindigni A, et al. The Na+ binding site of thrombin. *J Biol Chem*. 1995;270(38):22089–92.

[7] Sun WY, Smirnow D, Jenkins ML, et al. Prothrombin Scraton: substitution of an amino acid residue involved in the binding of Na+ (LYS-556 to THR) leads to dysprothrombinemia. *Thromb Haemost*. 2001;85(4):651–654.

[8] Pozzi N, Chen R, Chen Z, et al. Rigidification of the autoysis loop enhances Na+ binding to thrombin. *Biophys Chem*. 2011;159(1):6–13.

[9] Diuguid DL, Rabiet MJ, Furie BC, et al. Molecular defects of factor IX Chicago-2 (Arg145→His) and prothrombin Madrid (Arg271→Cys): arginine mutations that preclude zymogen activation. *Blood*. 1989;74(1):193–200.

[10] Bezaud A, Guillin MC, Olmeda F, et al. Prothrombin Madrid: A new familial abnormality of prothrombin. *Thromb Res*. 1979;16(1–2):47–58.

[11] James HL, Kim DJ, Zheng DQ, et al. Prothrombin Padua I: incomplete activation due to an amino acid substitution at a factor Xa cleavage site. *Blood Coagul Fibrinolysis*. 1994;5(5):841–844.

[12] O’Marcaigh AS, Nichols WL, Hassinger NL, et al. Genetic analysis and functional characterization of prothrombins Corpus Christi (Arg382-Cys), Dhahran (Arg271-His), and hypoprothrombinemia. *Blood*. 1996;88(7):2611–2618.

[13] Lefkowitz JB, Haver T, Clarke S, et al. The prothrombin Denver patient has two different prothrombin point mutations resulting in Glu-300Lys and Glu-309Lys substitutions. *Br J Haematol*. 2000;108(1):182–187.

[14] Akhavan S, Roche E, Zeinali S, et al. Gly319→Arg substitution in the dysfunctional prothrombin Segovia. *Br J Haematol*. 1999;105(3):667–669.

[15] Sun WY, Burkart MC, Holohan JR, et al. Prothrombin San Antonio: a single amino acid substitution at a factor Xa activation site (Arg320 to His) results indysprothrombinemia. *Blood*. 2000;95(2):711–714.

[16] Bafunno V, Bury L, Tiscia GL, et al. A novel congenital dysprothrombinemia leading to defective prothrombin maturation. *J Thrombosis Res*. 2014;134(5):1135–41.

[17] Morishita E, Saito M, Kabumashiri I, et al. Prothrombin Himi: a compound heterozygote for dysfunctional prothrombin molecules (Met-337→Thr and Arg-388→His). *Blood*. 1992;80(9):2275–2280.

[18] Miyata T, Morita T, Inomoto T, et al. Prothrombin Tokushima, a replacement of arginine-418 by tryptophan that impairs the fibrinogen clotting activity of derived thrombin Tokushima. *Biochemistry*. 1987;26(4):1117–1122.

[19] Lefkowitz JB, Weller A, Nuss R, et al. A common mutation, Arg457→Gln, link prothrombin deficiencies in the Puerto Rican population. *J Thromb Haemost*. 2003;1(11):2381–2388.

[20] Miyata T, Aruga R, Umeyama H, et al. Prothrombin Salakta: substitution of glutamic acid-466 by alanine reduces the fibrinogen clotting activity and the esterase activity. *Biochemistry*. 1992;31(33):7457–7462.

[21] Lefkowitz JB, Weller A, Nuss R, et al. A common mutation, Arg457→Gln, link prothrombin deficiencies in the Puerto Rican population. *J Thromb Haemost*. 2003;1(11):2381–2388.

[22] Miyata T, Aruga R, Umeyama H, et al. Prothrombin Salakta: substitution of glutamic acid-466 by alanine reduces the fibrinogen clotting activity and the esterase activity. *Biochemistry*. 1992;31(33):7457–7462.

[23] Stanchev H, Philips M, Villoutreix BO, et al. Prothrombin deficiency caused by compound heterozygosity for two novel mutations in the prothrombin gene associated with a bleeding tendency. *Thromb Haemost*. 2006;95(1):195–198.

[24] Sekine O, Sugo T, Ebisawa K, et al. Substitution of Gly-548 to Ala in the substrate binding pocket of prothrombin Perija leads to the loss of thrombinproteolytic activity. *Thromb Haemost*. 2002;87(2):282–287.

[25] Rouy S, Vidaud D, Alessandri JL, et al. Prothrombin Saint-Denis: a natural variant with a point mutation resulting in Asp to Glu substitution at position 552 in prothrombin. *Br J Haematol*. 2006;132(6):770–773.

[26] Henriksen RA, Mann KG. Substitution of valine for glycine-558 in the congenital dysthrombin thrombin Quick II alters primary substrate specificity. *Biochemistry*. 1989;28(5):2078–2082.