A natural variant with a point mutation resulting in a homozygous Arg to His substitution at position 388 in prothrombin

Prothrombin deficiency is probably the rarest inherited bleeding disorder with two phenotypes: (i) hypoprothrombinemia, with a concomitant decrease in prothrombin antigen and activity; (ii) dysprothrombinemia, with a decrease in prothrombin activity but normal or sub-normal antigen levels. Bleeding manifestations, usually absent in heterozygotes, vary in homozygotes and compound heterozygotes from very mild bleeding to life threatening hemorrhages. In hypoprothrombinemia, the severity of the bleeding manifestations is generally related to the prothrombin level. By contrast, this relationship is much more elusive in dysprothrombinemia and could be related to the molecular defect.[1] We report an homozygous mutation in the prothrombin gene ($Arg388_{(73)}His$, human prothrombin numbering with the chymotrypsinogen numbering of thrombin residues in brackets) and describe its phenotypic expression.

The study was approved by the local ethics committee and informed consent was obtained from the propositus and her parents prior to venipuncture. Plasma prothrombin assays included: (i) the classical one stage assay; (ii) a prothrombin assay with *Echis carinatus* venom (ECV) as activator and either fibrinogen or S 2238 as thrombin substrates; (iii) a quantitative Laurell electroimmunoassay. Prothrombin was isolated from plasma[2] and its conversion to thrombin was monitored in 10 mM Hepes, pH 7.5, 150 mM NaCl containing 1% PEG 8000, after adding 5 μM phospholipids vesicles, 20 pM bovine factor Xa, 20 pM human factor Va and 10 mM $CaCl_2$. At timed intervals, aliquots were analyzed by SDS PAGE and immunoblotting.

Thrombin was isolated by heparin-sepharose chromatography[3] after prothrombin activation with ECV. The initial rates of S 2238 hydrolysis by thrombin (0.5 nM) were measured using at least 10 different substrate concentrations (3 to 40 μM), in 20 mM Tris, 150 mM NaCl (TBS), pH 7.5, 0.1% PEG 8000 in the absence and presence of benzamidine. A Lineweaver Burk plot was constructed in the absence of an inhibitor at a fixed concentration of benzamidine, and the value of the inhibition constant (K_i) was determined. Thrombin activation of bovine protein C (PC) was measured in the absence and presence of rabbit thrombomodulin.[3] Genomic DNA was isolated from the blood leucocytes, and the coding region, intron/exon boundaries as well as the 5' and 3' untranslated region (UTR) of the prothrombin gene were amplified by PCR using 32 oligonucleotide primers, purified and sequenced on an ABI PRISM® 3130 DNA Sequencer (Applied Biosystems, Applera France SA).[4]

The proband was a 5 year old female who had been referred to us because a systematic blood coagulation evaluation revealed a prolonged prothrombin time and APTT, with an isolated prothrombin deficiency. The severe decrease in prothrombin activity (Table 1) demonstrated by clotting assays contrasted with subnormal levels of prothrombin measured by chromogenic assay and immunoassay. No consanguinity was reported in the family in the two preceding generations, but the father and mother, who originated from the same region of Turkey, presented with subnormal prothrombin activity (clotting assay) and normal prothrombin antigen. No abnormal bleeding has been observed in the proband, except sporadic post traumatic ecchymosis. The parents were asymptomatic. DNA sequence analysis of all 14 exons identified a missense mutation in exon 10, where G replaced an A at nucleotide g.15478 (GenBank accession number M17262), resulting in Arg $388_{(73)}$ substitution by His in the prothrombin molecule, designated FII-$R388_{(73)}H$. The proband was homozygous and both parents heterozygous for the mutation.

This mutation has previously been identified[5] in a compound heterozygote with two different mutations ($Met337_{(32)}Thr$ and $Arg388_{(73)}His$). However, the specific impact of $R388_{(73)}H$ substitution could not be studied in the compound heterozygote reported by Morishita *et al.*,[6] and has therefore been explored in the present study.

The rate of FII $R388_{(73)}H$ activation by prothrombinase was normal when examined by SDS PAGE (*data not shown*), indicating that the mutation does not significantly affect prothrombin activation. Hydrolysis of the synthetic substrate S 2238 by $R388_{(73)}H$-thrombin was normal (Km: 6.0±0.6 μM *vs.* 6.3±1.5 μM for normal thrombin) and the Ki for thrombin inhibition by benzamidine was normal (Ki: 2.8±0.15 mM *vs.* 2.9±0.3 mM for normal thrombin), indicating that $R388_{(73)}H$-thrombin has a normal catalytic site. Consistent with these observations, the rate of $R388_{(73)}H$-thrombin inhibition by antithrombin was only slightly reduced (17×10^4 mol-1 min-1 *vs.* 35×10^4 mol-1 min-1 for normal thrombin). The severe decrease in clotting activity of $R388_{(73)}H$-thrombin (10% of normal) was associated with a severe decrease in the initial rate of PC activation by the thrombomodulin/$R388_{(73)}H$-thrombin complex (0.5×10^{-2} mol APC min 1 mol–1 *vs.* 14.3×10^{-2} mol APC min–1 mol–1 for normal thrombin), consistent with the location of the mutation in thrombin exosite 1. In addition, the rate of PC activation in the absence of thrombomodulin was modestly reduced (1.6×10^{-4} mol APC min–1 mol–1 for $R388_{(73)}H$-thrombin *vs.* 5.8×10^{-4} mol APC min–1 mol–1 for normal thrombin). Since exosite 1 is not involved in thrombin interaction with PC, this may be the result of an allosteric effect exerted by the mutation.

Selective directed-mutagenesis has shown that

Table 1. Plasma prothrombin levels for the proband and her parents.

	Proband	Mother	Father	Normal range
Prothrombin antigen (%)	64	100	97	72-130
Prothrombin activity (%)				
clotting assay				
activator: prothrombinase	12	65	73	70-125
activator: ECV	11	nd	nd	70-125
chromogenic assay				
activator: ECV	60	nd	nd	70-130

ECV: Echis carinatus venom; nd: not determined.

every residue located within exosite 1 is not necessarily significant for all the interactions that occur at the site.[7-10] The natural R388(73)H thrombin variant reported here shows a severe defect in fibrinogen clotting and protein C activation. The Arg to His mutation exchanges a strongly positive amino acid for a weakly positive amino acid at neutral pH. As a consequence, the R388(73)H substitution has less dramatic consequences compared to the charge reversal substitution to Glu reported by Wu *et al.*,[7] but is very similar to the effect of the Arg to Ala mutation previously reported in Ala scanning mutagenesis studies.[8,9]

In conclusion, the experimental data showed that both procoagulant and anticoagulant functions of the R388(73)H natural variant are impaired. Akhavan *et al*.[1] have already proposed that substitutions that affect both procoagulant and anticoagulant functions of thrombin might, at least in part, counterbalance each other so that the hemostatic equilibrium is not drastically modified. The very mild bleeding tendency observed in the proband described here gives further support to this hypothesis.

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Key words: prothrombin deficiency, dysprothrombinemia, thrombin, exosite 1.

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Citation: d'Audigier C, Pasmant E, Bournier O, Laurian Y, Guillin MC, Bezeaud A. A natural variant with a point mutation resulting in a homozygous Arg to His substitution at position 388 in prothrombin. Haematologica 2008 May; 93(5):799-800.
doi: 10.3324/haematol.12520