平成 24 年度学位申請論文

Thrombosis From a Prothrombin Mutation Conveying Antithrombin-Resistance

（アンチトロンビン抵抗性を示す遺伝性プロトロンビン変異に起因する血栓症）

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主論文の要旨

【緒言】

遺伝性血栓症の患者には、しばしば若年時における静脈血栓症のエピソードや非定型血管への再発を呈し、多くに家族歴が見られる。遺伝性血栓性素因の遺伝的研究により、2 つの遺伝的異常が明らかにされている。1 つは自然抗凝固因子であるアンチトロンビン、プロテイン C および S における機能喪失型の遺伝子変異であり、もう 1 つは Factor V Leiden や Prothrombin G20210A に見られる血液凝固因子の機能獲得型遺伝子変異である。現在までに非常に多くの遺伝子異常が遺伝性血栓性素因を持つ家系から同定されたが、原因を推定できない症例も多くある。ここに我々は、血液凝固因子であるプロトロンビンをコードする遺伝子（*F2* ）に生じた変異「Prothrombin Yukuhashi」により、その活性型であるトロンビンがアンチトロンビン抵抗性を獲得し、血栓形成を誘発する新規メカニズムにより血栓症を発症した 1 家系について解析したので報告する。

【対象】

患者は日本人女性で 11 歳の時に深部静脈血栓症を発症した。患者家系は原因不明の血栓症家系で、3 世代に渡り 8 人の VTE 患者がみられ、うち 3 名はすでに亡くなっている。これまでに、この家系に対し遺伝性 VTE が疑われ、既知の先天性血栓性素因の調査がされたが全て否定された報告がある。

【方法】

本研究は名古屋大学医学部倫理委員会の承認もと、インフォームドコンセントを得て実施した。

まず、ダイレクトシークエンス法により、*F2* を検索し遺伝子変異を同定した。次に、分子生物学的手法を用い、ヒト胎児腎由来細胞株（HEK293）において野生型、変異型のリコンビナントプロトロンビンを作成した。

始めに、ウシ活性化第 X 因子（FXa）によるプロトロンビナーゼを用いて、プトロンビンからトロンビンへの活性化動態をウェスタンブロット法にて確認した。次いで、凝固 1 段法、凝固 2 段法および発色基質法の 3 法を用いて、野生型と変異型の凝固能を比較した。さらに、トロンビン・アンチトロンビン複合体（TAT）の形成率を ELISA 法にて測定した。最後に、トロンビンジェネレーションアッセイ（TGA）を用いて、同定したプロトロンビン変異が血漿検体における組織因子をトリガーとした凝固反応に及ぼす影響、すなわち生成トロンビン活性の推移動態について検討した。TGA では、対照をノーマルプールプラズマとし、欠乏血漿を利用して疑似野生型ホモ（疑似健常血漿）、疑似ヘテロ（疑似患者血漿）、変異型ホモ（疑似ホモ接合体血漿）に、AT50%（疑似 AT 欠損血漿）を加えた 5 種類のサンプルを作製し測定した。

【結果】

我々は患者の *F2* エクソン 14 内に 1787 番目のグアニンがチミンに変わる 1 塩基置換をヘテロ接合体として同定した。野生型、変異型リコンビナントプロトロンビンを作成したが発現量に差は見られず、また、ウシ FXa による活性化動態を確認したところ、両者に差は見られなかった。

3 種の凝固法による凝固活性測定の結果、野生型は全て同様の値を示したのに対し、変異型は全ての測定法で野生型を下回り、その活性は凝固 1 段法、凝固 2 段法、発色基質法の順に大きくなった。次に、ELISA 法による TAT の形成率は、野生型が経時的な上昇を示したのに対し、変異型では 30 分までは検出限界未満、60 分後でも野生型の約 4%という極めて低い値を示した。TGA ではノーマルプールプラズマと疑似健常血漿は同様のパターンを示したが、変異型プロトロンビンを含む疑似患者血漿と疑似ホモ接合体血漿では、ピークの低下に加え、ピーク後に特徴的な緩やかな下降を示した。疑似 AT 欠損血漿ではピークは野生型同様であったが、ピーク後に緩やかな下降を示した。AT を添加した補正試験では、疑似 AT 欠損血漿は疑似健常血漿と同様のパターンに補正されたが、疑似患者血漿と疑似ホモ接合体血漿はピーク後の緩やかな下降は補正されなかった。

【考察】

患者から同定された *F2* の c.1787G>T 変異は、トロンビンのカタリティックドメイン内に位置する 596 番目のアミノ酸であるアルギニンをロイシンに変化させる、これまでに報告のない新規のミスセンス変異であった。この変異は、患者の母親および家系内 3 人の VTE 患者からも同定されたことから、本家系における血栓症の原因であると考えられた。また、596 アルギニンは AT との結合領域の 1 つであるナトリウムイオン結合領域内に存在し、また AT 分子の 265 アスパラギンとの直接的な水素結合などに関与しており、ロイシンへの置換によりナトリウムイオン結合領域の不安定化が起こることが予想された。

我々は患者家系の臨床状態から、変異型由来トロンビンは凝固能を保っていること、そして AT との結合障害により、不活化を受けにくい AT 耐性トロンビンを生ずること、この２つにより残存トロンビン活性が血中で増加し、患者家系における遺伝性血栓症の原因であると仮説を立てた。

変異型プロトロンビンが凝固能を保っているという仮説の検証のため、3 種の活性測定試験で検討を行ったところ、凝固 1 段法より凝固 2 段法での活性がやや上昇したことから、変異型ではプロトロンビンからトロンビンへの活性化がやや遅延していることが示唆された。また、凝固 2 段法の結果から、変異型由来トロンビンはフィブリノゲンに対する活性が野生型の約 30%であることが示唆された。これは、変異型由来トロンビンでは、野生型と比較してナトリウムイオン結合領域に構造変化が起こり、その活性低下を招いたためと考えられた。しかし、凝固 2 段法より発色基質法での活性が上昇したことから、完全に機

能を失うほどの立体構造変化ではなく、フィブリノゲンのような大きな基質の認識・捕捉は悪くなっているものの、特に活性中心の構造はある程度保たれ、変異型由来トロンビンは野生型の約 30%とはいえ、凝固能を保持していることが示唆された。

続いて、2 つ目の仮説、変異型由来トロンビンは AT による不活化に耐性を示すか否かを検証するため、ELISA 法による結合実験を行った。その結果、60 分後でも TAT の形成が極めて低く、変異型由来トロンビンは AT との結合が著しく障害されていることが確認された。

最後に、TGA による凝固反応のグローバル評価では、疑似患者血漿および疑似ホモ接合体血漿は、両者ともピークが低く、凝固能の低下を示した。一方で、ともにピーク後の下降がゆるやかであり、不活化不良を示唆する結果だった。同様に不活化不良が予測された疑似 AT 欠損血漿は、ピークは疑似健常血漿と変わらなかったが、ピーク後の下降は緩やかであり、やはり不活化不良を示す結果となった。AT 添加の補正試験において、変異型プロトロンビンを含むサンプルで、ピーク後の緩やかな下降が補正されなかったことは、変異由来トロンビンが AT との結合能低下を示したことと合わせて、この異常プロトロンビン血症での AT 抵抗性を示す結果であった。

【結語】

変異型プロトロンビンはトロンビンへの活性化が野生型よりわずかに遅く、またフィブリノゲンに対する活性も野生型の約 30%と低いことが判明した。生成されたトロンビンは、主に AT と結合し TAT を形成して不活化されるが、変異型由来トロンビンは AT との結合障害・不活化不全を示す AT 耐性トロンビンであり、低いながらも凝固能を持った変異型由来トロンビンが血中にとどまり、残存トロンビン活性が増加することで、患者家系において遺伝性血栓性素因の病態を示すことが考えられた。

これらの結果は、抗凝固因子の異常により血栓性素因が生ずるという、従来の一般的な概念に反するものである。凝固因子であるプロトロンビンの異常症は多くは出血傾向を示すとされて来たが、Prothrombin Yukuhashi は 1 アミノ酸の変化にも関わらず、逆に血栓症を誘発することが同定された世界ではじめての病態解析報告である。

主論文

Thrombosis from a Prothrombin Mutation Conveying Antithrombin Resistance

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Summary

We identified a novel mechanism of hereditary thrombosis associated with anti-thrombin resistance, with a substitution of arginine for leucine at position 596 (p.Arg596Leu) in the gene encoding prothrombin (called prothrombin Yukuhashi). The mutant prothrombin had moderately lower activity than wild-type prothrombin in clotting assays, but the formation of thrombin–antithrombin complex was substantially impaired. A thrombin-generation assay revealed that the peak activity of the mutant prothrombin was fairly low, but its inactivation was extremely slow in reconstituted plasma. The Leu596 substitution caused a gain-of-function mutation in the prothrombin gene, resulting in resistance to antithrombin and susceptibility to thrombosis.

Introduction

Patients with hereditary thrombophilia often present with unusual clinical episodes of venous thrombosis at a young age and recurrence in atypical vessels, often with a family history of the condition [1]. Genetic studies of hereditary thrombophilia have revealed two types of genetic defects : loss-of-function mutations in the natural anticoagulants antithrombin, protein C, and protein S, along with gain-of-function mutations in procoagulant factors V (factor V Leiden) and II (prothrombin G20210A) [2]. To date, numerous genetic defects have been found in families with hereditary thrombophilia, but there may be many undiscovered causative mutations [3]. Here, we describe a case of hereditary thrombosis induced by a novel mechanism of antithrombin resistance, a gain-of-function mutation in the gene encoding the clotting factor prothrombin (prothrombin Yukuhashi).

Case Report

The proband was a 17-year-old Japanese girl who had a first episode of deep-vein thrombosis at the age of 11 years and had since been treated with warfarin. Her family originated in Yukuhashi in the northern part of the Kyushu islands. At least nine of her family members had had one or more episodes of deep-vein thrombosis (Fig. 1A), including two with pulmonary embolism and three who died from thrombosis. Five family members, including the proband, had had juvenile thrombosis, with two reporting episodes during early childhood. Previous studies did not identify any known causes of hereditary thrombophilia in this family [4].

Methods

DNA Analysis

We amplified all 14 exons, including the exon–intron boundaries and the 3′ untranslated region, of the prothrombin gene by means of polymerase chain reaction (PCR), using gene-specific primers (see Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). The amplicons were sequenced as described previously [5]. To detect the mutation, we performed PCR–restriction-fragment–length polymorphism (RFLP) analysis, using a mismatched lower primer (5′-TGTAGAAGCCATATTTCCCcTgC-3′, with base substitutions at c and g) and introducing a PstI site into the amplicon from a mutant allele. Genomic DNA was isolated from peripheral leukocytes by phenol extraction [6].

Recombinant Prothrombins

We used a PCR assay to prepare full-length human prothrombin complementary DNA (cDNA) obtained from a human liver cDNA library (Clontech)

and cloned this into pcDNA^{TM} 3.1(+) (Invitrogen) to obtain a wild-type human prothrombin expression vector. Subsequently, we prepared a mutant prothrombin expression vector by means of overlap extension PCR [7], using two primers: 5′-TGAAGGCTGTGACCtGGATGGGAAA-3′ (sense primer with a base substitution at t) and 5′-TTTCCCATCCaGGTCACAGCCTTCA-3′ (antisense primer with a base substitution at a).

We transfected human embryonic kidney cells (HEK293) with the prothrombin expression vectors using the calcium phosphate method [8]. We established stable transformants by selection with G418 and determined which of these had high levels of prothrombin expression by means of a dot-blot immunoassay. Conditioned media of stable transformants expressing recombinant prothrombins in serum-free medium containing vitamin K were collected, concentrated, and stored at −80°C until use. We determined the antigen levels of the prothrombins using an enzyme-linked immunosorbent assay (ELISA, Enzyme Research Laboratories).

Functional Assays of Recombinant Prothrombins

We performed three tests of prothrombin activity: a one-stage clotting assay, a two-stage clotting assay, and a chromogenic assay that uses S-2238 (a thrombin

substrate that generates color at the time of cleavage). In the latter two assays, we used *Oxyuranus scutellatus* venom (Sigma Aldrich) as a factor Xa–like enzyme. To examine the functions of the recombinant prothrombins in plasma, we prepared reconstituted plasma by mixing prothrombin-deficient plasma (prothrombin activity, <1%; Mitsubishi Chemical Medience) with the recombinant prothrombins on the assumption that the prothrombin concentration was 100 μg per milliliter in normal plasma (100%) [9]. The proband's plasma was not suitable for evaluation because of warfarin treatment.

Formation of Thrombin–Antithrombin Complex

To evaluate the ability of the wild-type and mutant recombinant prothrombins to form complexes with antithrombin, we converted the recombinant prothrombins to thrombins, using bovine factors Xa (Haematologic Technologies) and Va (Thermo Scientific), cephalin (Roche Diagnostica Stago), and calcium chloride. We then incubated the thrombins with human antithrombin (Mitsubishi Tanabe Pharma), with or without unfractionated heparin (Mochida Pharmaceutical), at 37°C for various time periods. The reactions were stopped with PPACK (d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone) (Calbiochem), and thrombin–antithrombin complex formation

was measured with the use of the AssayMax Human TAT Complexes ELISA kit (Assaypro).

Thrombin-Generation Assay

We prepared wild-type, mutant, and heterozygous-mutant reconstituted plasma by mixing prothrombin-deficient plasma with the recombinant prothrombins, at a final prothrombin concentration of 100%, and by mixing antithrombin-depleted plasma (Affinity Biologicals) with human antithrombin, at a final antithrombin concentration of 50%. We used normal pooled plasma as a control. The thrombin-generation assay was performed by means of calibrated automated thrombography (CAT, Thrombinoscope BV), in accordance with the manufacturer's instructions. We monitored the reactions for 2 hours, using Fluoroscan Ascent FL (Thermo LabSystems), set at an excitation wavelength of 390 nm and an emission wavelength of 460 nm, and Thrombinoscope software (Thrombinoscope BV).

Study Oversight

The study was approved by the ethics committee at the Nagoya University

School of Medicine. Written informed consent was obtained from all study participants.

Results

DNA Analysis

Genomic DNA analysis of the proband revealed that she was heterozygous for a novel missense mutation in the prothrombin gene (c.1787G→T, p.Arg596Leu) (Fig. 1B). The nucleotide and protein numbering system is based on the nomenclature recommended by the Human Genome Variation Society [10]. The same mutation was detected in her mother and in three other family members with deep-vein thrombosis but not in an asymptomatic family member. On mismatch PCR-RFLP analysis, the amplicon that was treated with PstI displayed a 192-bp band (mutant allele) and a 212-bp band (normal allele). We confirmed the heterozygosity of this mutation in the proband, her mother, and three other family members with deep-vein thrombosis but not in an asymptomatic family member (Fig. 1C). We did not detect the mutation in samples obtained from 100 Japanese persons with a normal phenotype and in 5 persons with undiagnosed thrombosis before this testing.

Recombinant Prothrombins

We established stable transformants of HEK293 cells expressing the wild-type and mutant prothrombins. To evaluate γ-carboxylation of the recombinant prothrombins, we used ELISA to measure prothrombin levels in the culture medium after barium sulfate absorption. We found that both the wild-type and mutant prothrombins were completely absorbed, suggesting that appropriate γ-carboxylation occurred in both preparations (data not shown).

Functional Assays of Recombinant Prothrombins

We performed three assessments of recombinant prothrombin activity: one-stage clotting, two-stage clotting, and chromogenic assays (Table 1). Reconstituted plasma was used in all tests. Values for the wild-type recombinant prothrombin were approximately 100% in all assays. The mutant prothrombin activity in the one-stage assay was lower than that in the two-stage assay. The mutant prothrombin activity in the chromogenic assay was higher than that in the two-stage assay.

Formation of Thrombin–Antithrombin Complex

We used ELISA to determine whether there was a difference between the

wild-type and mutant prothrombins in forming thrombin–antithrombin complexes. The recombinant prothrombins that were activated by factor Xa were incubated with antithrombin, and thrombin–antithrombin complex formation was determined by means of ELISA. In the absence of heparin, thrombin–antithrombin complex formation by the wild-type prothrombin increased in a time-dependent manner. However, thrombin–antithrombin complex formation by the mutant prothrombin was almost negligible for the first 30 minutes (Fig. 1D). In the presence of heparin, thrombin–antithrombin complex formation was greatly increased in both samples but remained substantially impaired in the mutant sample.

Thrombin-Generation Assay

A thrombin-generation assay was performed to evaluate the effect of the mutation on thrombin generation in plasma (Fig. 2). The values for wild-type reconstituted plasma were similar to those for normal plasma, but the mutant plasma showed a decreased maximum concentration of thrombin (peak), an extension of the total duration of thrombin-generation activity (start tail), and increased thrombin activity, which was assessed as the area under the curve for endogenous thrombin potential. The

heterozygous-mutant plasma, mimicking the proband's plasma, showed intermediate values. The 50% antithrombin plasma, mimicking the antithrombin-deficient plasma, showed similar changes (except for a decreased peak), which were canceled by the addition of human antithrombin at a final concentration of 150%. These data indicate that the thrombin activity derived from the mutant prothrombin was lower than that derived from the wild-type prothrombin, but its inactivation was exceedingly slow, resulting in a prolonged procoagulant state in the proband's plasma.

Discussion

Numerous gene mutations in various molecules have been found in members of families with inherited thrombophilia, but many mutations remain unidentified [3]. The G20210A mutation in the prothrombin gene is associated with a mild risk of thrombosis in the white population, but many other prothrombin gene mutations lead to bleeding tendencies, such as prothrombin deficiencies, dysprothrombinemia, and hypoprothrombinemia [11-13]. A genomewide analysis to detect genes that are associated with a susceptibility to thrombosis also identified a prothrombin gene mutation, but the detailed molecular mechanism for inherited thrombophilia remains unknown [14]. In this study, we investigated possible causative genetic defects in samples obtained from a large Japanese family with inherited thrombophilia. We found a novel missense mutation in the prothrombin gene (p.Arg596Leu) that resulted in a variant prothrombin (prothrombin Yukuhashi). The mutation cosegregated with deep-vein thrombosis in this family, indicating that it could be a cause of hereditary thrombophilia.

Thrombin, which is an active form of prothrombin, is an allosteric enzyme

controlled by the binding of sodium [15, 16]. Sodium-bound thrombin (known as the fast form) is optimized for procoagulation because of its increased substrate specificity for fibrinogen, whereas sodium-free thrombin (known as the slow form) is an anti-coagulant because of its increased specificity for cleaving protein C. The mutation occurred at residue Arg596 (Arg221a in the chymotrypsinogen numbering system [17]) within the sodium-binding region of thrombin and was expected to have an effect on sodium binding. The mutation is also located at one of the antithrombin-binding sites where thrombin is inactivated by antithrombin with heparin.18 Two exosites on thrombin, the γ-loop and the sodium-binding region, are critical for stabilizing a thrombin–antithrombin complex [18] (Fig. S1A in the Supplementary Appendix). Two hydrogens of the Arg596 side chains of thrombin form hydrogen bonds with oxygen of the Asn265 side chain of antithrombin (Fig. S1B in the Supplementary Appendix). Therefore, we propose two hypotheses: first, that the procoagulant activity of the mutant prothrombin is somewhat impaired; and second, that complex formation involving the mutant thrombin and antithrombin is impaired, resulting in prolonged residual thrombin activity.

To test the first hypothesis, we examined the activation and procoagulant functions of the recombinant prothrombins. We prepared reconstituted plasma by

mixing prothrombin-deficient plasma with the recombinant prothrombins, since the proband's plasma was not suitable for evaluation because of warfarin treatment. We observed that the mutant and wild-type prothrombins were fully converted to thrombins in a similar manner by prothrombinase within 5 minutes (Fig. S2 in the Supplementary Appendix). However, conversion of the mutant prothrombin to thrombin appeared to be a few seconds slower than that of the wild-type thrombin in the clotting assays. In addition, the mutant thrombin probably had a lower catalytic activity for fibrinogen than did the wild-type thrombin, which may have been the result of structural disruption of the sodium-binding region by the Leu596 substitution for Arg. In a previous study of alanine-scanning mutagenesis, thrombin with an Ala596 mutation showed a reduction by a factor of 5 in sodium-binding affinity, and its procoagulant activity was similar to that of the slow form of thrombin [19]. Similar mechanisms of structural disruption in the Leu596 mutant thrombin may have resulted in lower catalytic activity for fibrinogen.

To test the second hypothesis — that the mutant thrombin would be defective in terms of its interaction with antithrombin — we examined thrombin–antithrombin complex formation using ELISA. The mutant thrombin sample had extremely low levels of thrombin–antithrombin complex formation. This suggests that the disruption of

the sodium-binding region, which resulted in the loss of two hydrogen bonds between Arg596 of thrombin and Asn265 of antithrombin, may be critical for the formation of the thrombin–antithrombin complex. These findings indicate that prothrombin Yukuhashi can be characterized as a dysprothrombin that is highly resistant to inhibition by antithrombin.

We next performed a thrombin-generation assay to determine the potential procoagulant activity of the recombinant prothrombins in plasma. A thrombin-generation assay is a comprehensive coagulation-function test that allows evaluation not only of the initial phase of thrombin generation but also of the late phase of its inactivation. Data from this assay again suggested that the mutant prothrombin had low procoagulant activity but was highly resistant to antithrombin. Thus, its active form, the mutant thrombin, would not be inactivated by antithrombin and would continue to facilitate blood coagulation, despite its low activity level.

In conclusion, we identified a novel mechanism of hereditary thrombosis in a Japanese family, in which antithrombin resistance was associated with a missense mutation in the prothrombin gene (p.Arg596Leu). This mutation results in slightly impaired but adequate procoagulant function of the mutant prothrombin but considerably impaired inhibition of the mutant thrombin by antithrombin. The

antithrombin-resistant thrombin may have prolonged procoagulant activity in vivo, conferring a susceptibility to thrombosis.

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Table 1. Procoagulant and Amidolytic Activities of the Recombinant Prothrombins.*

Prothrombin	Antigen†	Activity‡		
		One-Stage Clotting Assay	Two-Stage Clotting Assay	Chromogenic Assay
	percent			
Wild-type	112	91	109	88
Mutant	118	15	32	66

* The values were measured from reconstituted plasma in prothrombin-deficient plasma. The value of normal plasma was assigned as 100%.

† The values for prothrombin antigens were determined by means of enzyme-linked immunosorbent assay.

‡ The prothrombin activities were determined by three methods: the classic one-stage clotting assay, in which thromboplastin is used; the two-stage clotting assay, in which *Oxyuranus scutellatus* venom (Ox) is used as a factor Xa–like enzyme and fibrinogen from pooled normal plasma is used as a substrate; and the chromogenic assay, in which Ox venom is used as an activator and S-2238 as a substrate.

Figure legends

Figure 1. Prothrombin Genotype of a Family with Hereditary Thrombophilia.

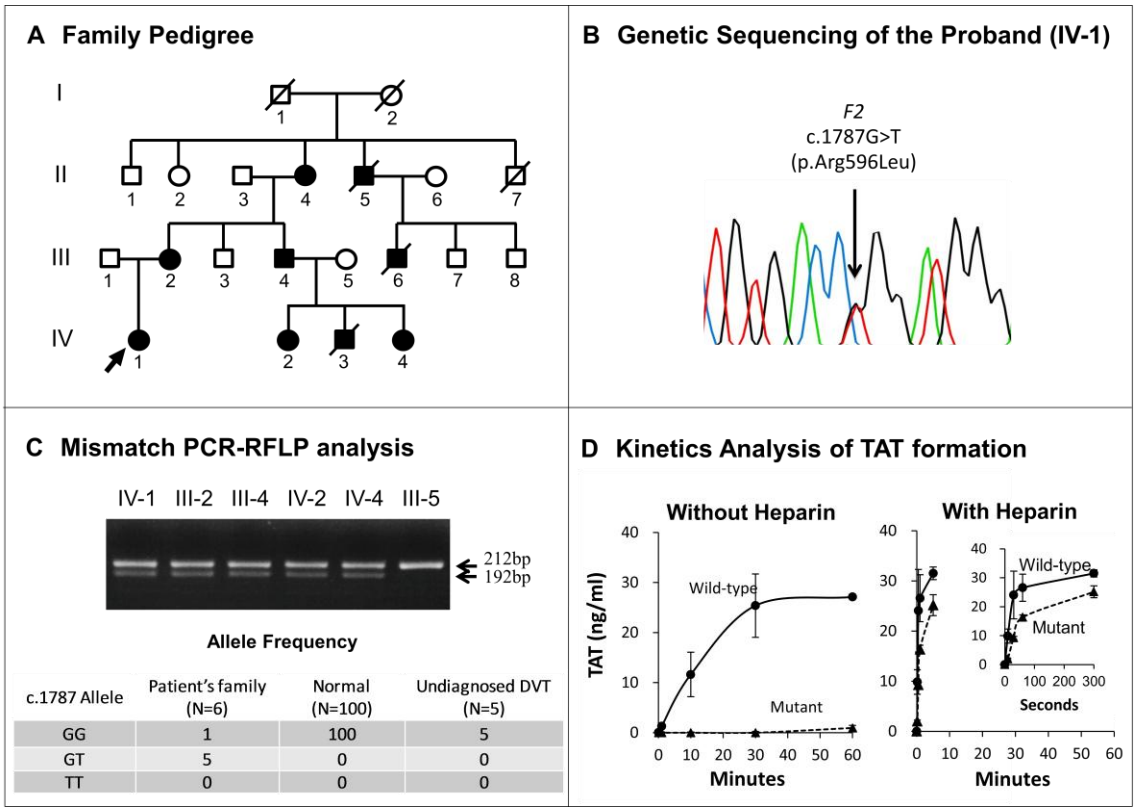
Panel A shows the family pedigree. The proband (IV-1) is indicated by an arrow. Solid symbols represent affected family members, open symbols unaffected family members, and slashed symbols deceased family members. Circles represent female family members, and squares male family members. Panel B shows the sequence of the prothrombin gene around the site of the mutation in exon 14. A G→T transversion at nucleotide 1787 of the coding sequence (c.1787G→T) has occurred in the gene encoding the clotting factor prothrombin Yukuhashi, resulting in an amino acid substitution of leucine for arginine at position 596 (p.Arg596Leu). The proband is heterozygous for the mutation (arrow). Panel C shows a restriction-fragment–length polymorphism (RFLP) analysis of the mismatch polymerase-chain-reaction (PCR) product of exon 14 of the prothrombin gene digested by PstI endonuclease. The wild-type prothrombin gene has an undigested fragment of 212 bp. The mutation in the prothrombin Yukuhashi gene creates a PstI site, resulting in a digestion fragment of 192 bp. The table shows allele frequencies at c.1787 of the prothrombin gene in 6 family members, in 100 Japanese persons with a normal phenotype, and in 5 persons with

previously undiagnosed deep-vein thrombosis (DVT). Panel D shows the kinetics analysis of thrombin–antithrombin complex (TAT) formation of recombinant wild-type and p.Arg596Leu mutant prothrombins, in the presence and absence of heparin. TAT levels were measured with the use of an enzyme-linked immunosorbent assay (ELISA) and various incubation times for antithrombin and recombinant thrombins; the latter are forms of recombinant prothrombins activated by factor Xa.

Figure 2. Thrombin-Generation Assays with and without Excess Antithrombin.

Panel A shows the results of a thrombin-generation assay of normal plasma as well as reconstituted plasma samples, with recombinant prothrombins in prothrombin-deficient plasma and of human antithrombin (AT 50%) in antithrombin-depleted plasma. The heterozygous-mutant (mutant-hetero) plasma contained 50% each of wild-type and mutant prothrombin. The table at the right shows the total amount of thrombin activity, which was assessed as the area under the curve for endogenous thrombin potential (ETP), the maximum concentration of thrombin (peak), and the total duration of thrombin-generation activity (start tail). Panel B shows the results of a thrombin-generation assay of the respective plasma samples after the addition of excess antithrombin.

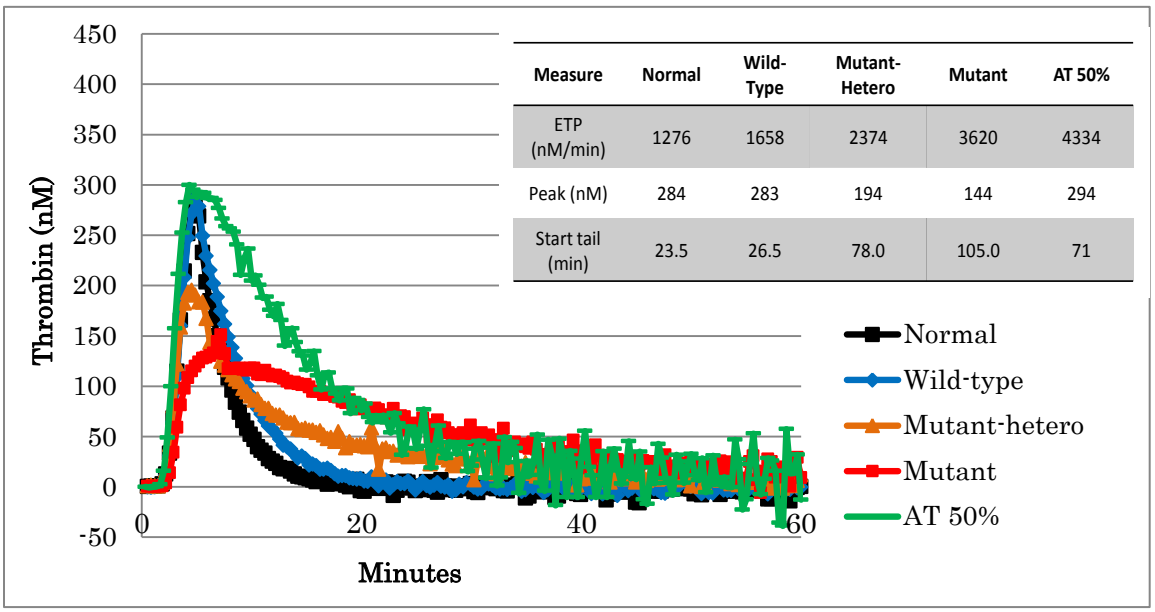
Figure 1. Prothrombin Genotype of a Family with Hereditary Thrombophilia.



c.1787 Allele	Patient's family (N=6)	Normal (N=100)	Undiagnosed DVT (N=5)
GG	1	100	5
GT	5	0	0
TT	0	0	0

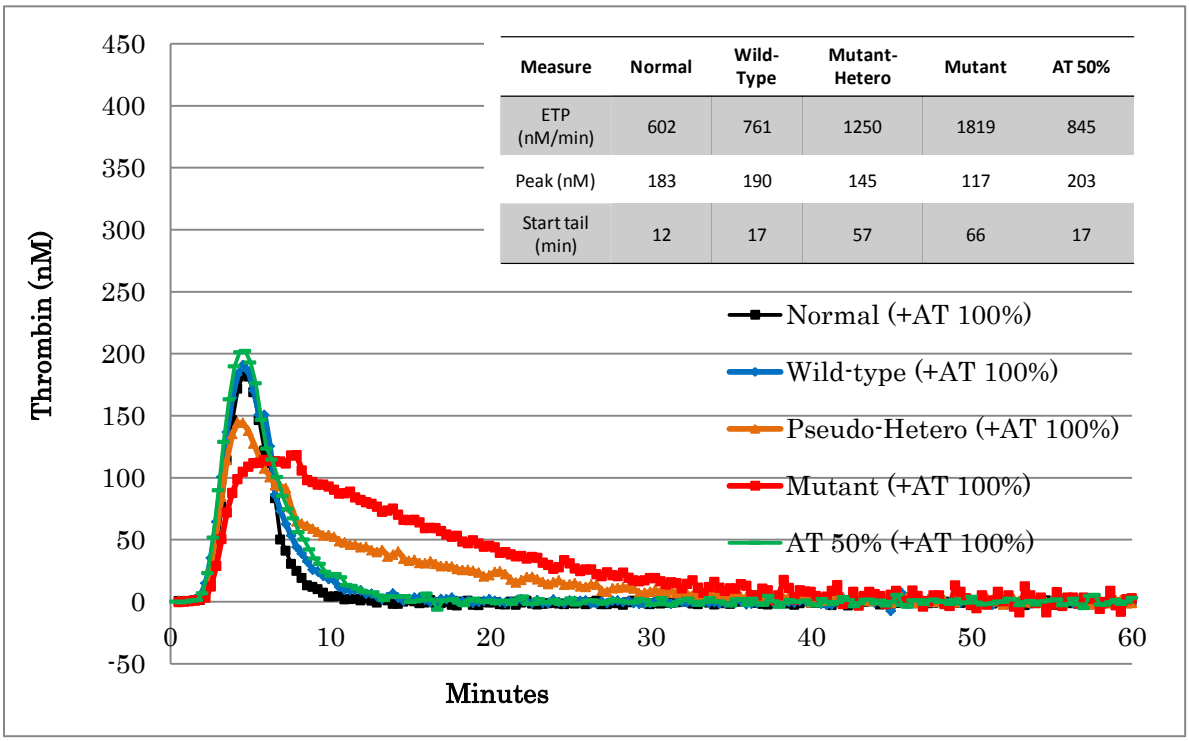
Figure 2. Thrombin-Generation Assays with and without Excess Antithrombin.

A. Thrombin Generation



Measure	Normal	Wild-Type	Mutant-Hetero	Mutant	AT 50%
ETP (nM/min)	1276	1658	2374	3620	4334
Peak (nM)	284	283	194	144	294
Start tail (min)	23.5	26.5	78.0	105.0	71

B. Thrombin Generation with Excess Antithrombin



Measure	Normal	Wild-Type	Mutant-Hetero	Mutant	AT 50%
ETP (nM/min)	602	761	1250	1819	845
Peak (nM)	183	190	145	117	203
Start tail (min)	12	17	57	66	17

Supplementary data

Table of contents

Suppl. Methods

Conversion of recombinant prothrombins by the prothrombinase complex

Wild-type and mutant recombinant prothrombins (80 nM) were treated with a prothrombinase complex containing bovine factor Xa (10 nM; Haematologic Technologies), bovine factor Va (10 nM; Thermo Scientific), Cephalin (10% (v/v); PTT-reagent RD, Roche Diagnostica Stago) and 2 mM CaCl2 in Tris-buffered saline and 0.01% (v/v) Tween-20 at 37°C. Reactions were initiated by the addition of factor Xa followed by the removal of aliquots at timed intervals. The samples were then separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions, and transferred to polyvinylidene difluoride membranes (Amersham Biosciences) for immunoblotting as described previously [1].

Suppl. Results

Conversion of the recombinant prothrombins by prothrombinase complex

Activation of prothrombin by the prothrombinase complex produced thrombin and varied derivatives2. The time courses of the activation patterns were similar in both recombinant prothrombins, as shown in Suppl. Fig. 1. Both prothrombin bands had almost disappeared after 5 min, demonstrating that the mutant prothrombin was proteolysed by the prothrombinase complex in a similar way to the wild-type prothrombin.

Suppl. Figure legends

Suppl. Fig. S1. Structural features of the thrombin-antithrombin complex (PDB ID: 1TB6).

Panel A shows the crystal structure of the thrombin-antithrombin complex with heparin (left) and that of the hidden heparin (right). Thrombin (light blue, light chain; white, heavy chain) and antithrombin (green) are combined via two exosites with heparin (violet stick), the γ-loop binding region, and the Na+ binding region (yellow circle). The blue residues are the active center of thrombin and the Arg596 of thrombin (arrowed yellow residue) is located away from the active center. The red residues of thrombin and the magenta residues of antithrombin are involved in thrombin-antithrombin complex formation.

Panel B shows Na+ binding region interactions. The side chain of Arg596 (yellow) in thrombin forms two hydrogen bonds (light blue dashed line) with the side chain of Asn265 in antithrombin. Glu264 of antithrombin also forms a salt bridge with Lys599 of thrombin involving a water-mediated hydrogen bond network with surrounding residues Thr540, Arg541, Glu592 and Lys5993. Residues of thrombin and antithrombin are shown in white and green, respectively. The water molecule is shown as a light blue

sphere.

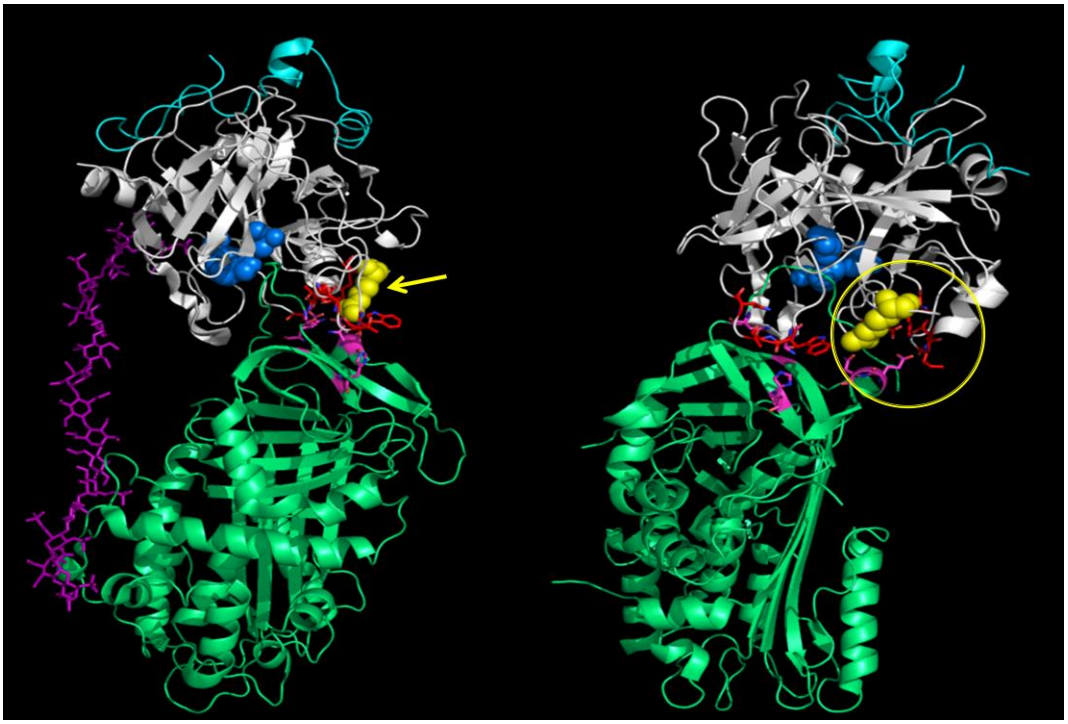
Suppl. Fig. S2. Conversion of recombinant prothrombins by prothrombinase.

Recombinant wild-type and mutant prothrombins were activated at 37°C with 10 nM of bovine factors Xa and Va, 10% phospholipid in TBS, 2 mM CaCl2, 0.01% (v/v) Tween-20, pH7.4. Aliquots of reaction mixtures were removed at the specified time intervals and analyzed by SDS-PAGE on 10% polyacrylamide gels before immunoblotting. The molecular weight markers are indicated on the left.

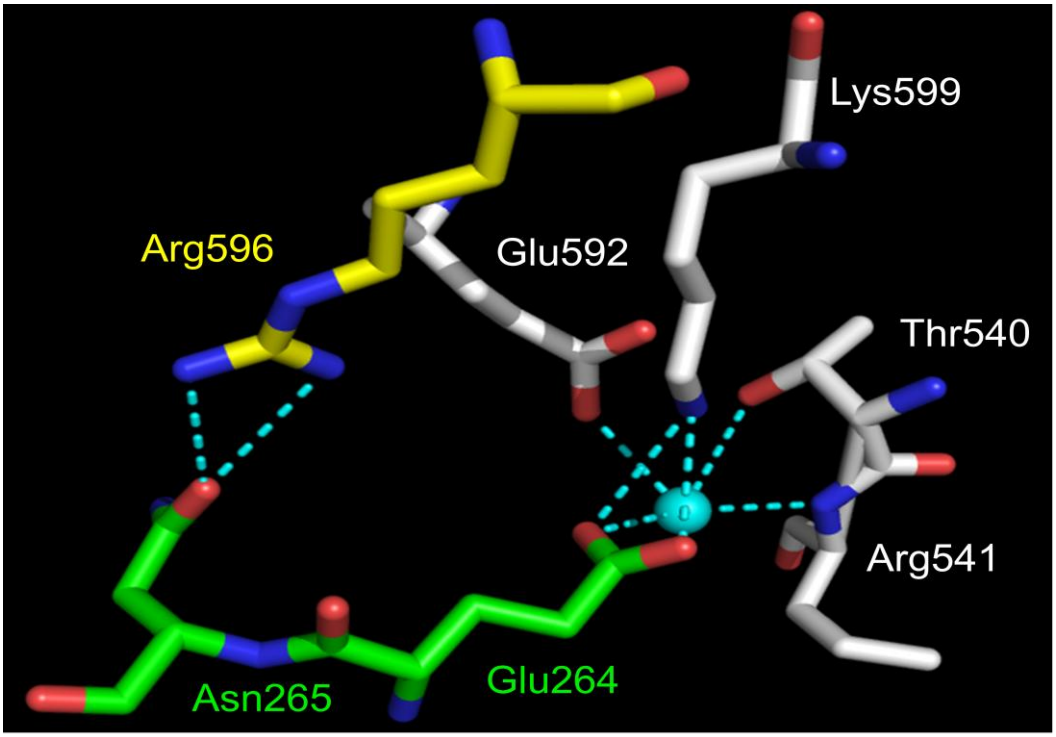
The prothrombin fragments shown are as follows: FII, prothrombin; F1.2, fragment 1.2; P2, prothrombin-2; TB, B chain of α-thrombin; and F1; fragment 1.

Suppl. Fig. S1. Structural features of the thrombin-antithrombin complex (PDB ID: 1TB6).

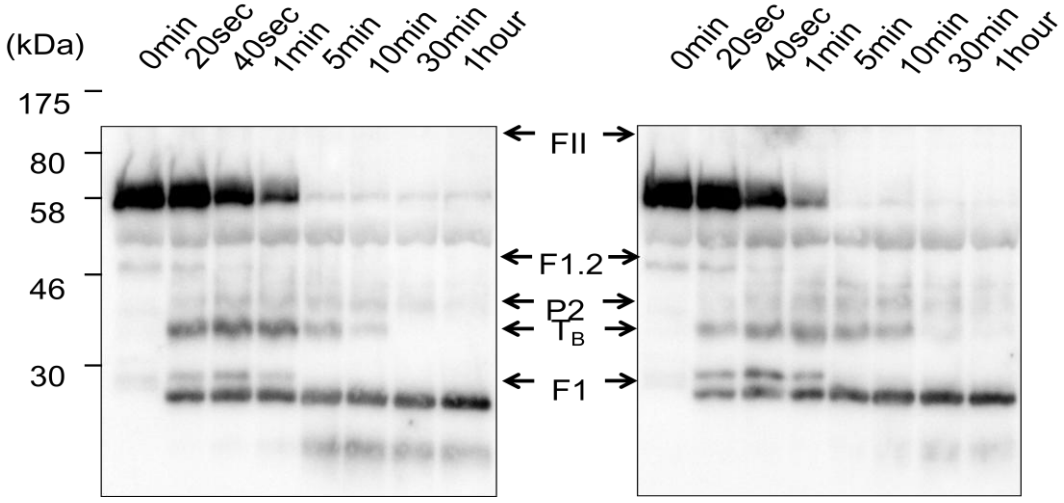
A



B



Suppl. Fig. S2. Activation of recombinant prothrombins by prothrombinase.



Suppl. Table S1. Primers for PCR amplification of the prothrombin gene.

exon		Oligonucleotide Sequence	Annealing (°C)	PCR Product (bp)
1	Up	TGGAGATGGACAGGAGGACT	60	337
	Lw	ACCTACTTAGGGGCCAGCTC		
2	Up	CCTCTCTCAGAAGCCAGCAG	60	388
	Lw	TGAAATGAGGCTGTGAGCAG		
3-4	Up	GCGTGACCAGGGTAAAGGAA	60	493
	Lw	AAACCCACCCCTGAGCTCTT		
5-6	Up	TGGGGGATAGACAACTTTGC	60	499
	Lw	TTCTTGGTTCCCATCCCAG		
7	Up	GTCACACAGGCAGAAAGCAG	60	489
	Lw	CAGAAGCGGCTGTTGTTATT		
8-9	Up	GATCTAGGGGATGGGTGAGG	60	461
	Lw	GGGTCCAGCAGCACACCT		
10	Up	GGGTTCTTAGACCTGGGATTG	60	320
	Lw	CATGATCGCTTTGGAGGACT		
11	Up	GCAGGACACACTGTCTCCCAGAC	60	368
	Lw	AAAAGGGAAAGGGGCTCTTGC		
12	Up	CCAGCTCTGGCGTTTTAGAT	60	400
	Lw	TGAGCCACCAAGAGGTTAGG		
13	Up	AAGTGGGGACAGCAAGAATGA	60	309
	Lw	GAGTCAAGTTCAAGGTCACATCAG		
14	Up	AGGGCCTGGTGAACACATCTTC	60	467
	Lw	CCAGGTGGTGGATTCTTAAGTCTTC		

Suppl. References

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