ELISA

BMP4 protein levels in the supernatants from transduced murine hematopoietic cells were determined by ELISA. Briefly, mCherry-positive bone marrow cells transduced with empty (MIC), GLIS2, or CBFA2T3-GLIS2-containing retroviruses were placed in media containing IL3, IL6, and SCF for 48 hr, and supernatant was then harvested and the level of murine BMP4 determined using an ELISA kit purchased from TSZELISA (http://www.tszelisa.com). Measurements were done according to manufacturer's instructions.

Transgenic *Drosophila*

CBFA2T3-GLIS2 and *GLIS2* cDNAs were subcloned into the *pUAS-attB* plasmid (Bischof et al., 2007). Transgenic UAS-*CBFA2T3-GLIS2* and UAS-*GLIS2* flies were generated using site-specific ϕC31 integration system (Bischof et al., 2007). Embryo injections were performed by Best Gene. UAS constructs were targeted to chromosome 2R-51D in order to avoid differential positional effects on transgene expression. For wing imaginal disc staining, relevant crosses were performed to generate flies carrying all three transgenes: *Apterous-Gal4* (a strong epithelial dorsal compartment-specific GAL4 driver), UAS-*CBFA2T3-GLIS2*, and a *dpp-lacZ* enhancer trap reporter. Gal4 driver and *dpp-lacZ* reporter stocks were obtained from the Bloomington Stock Center. Wing imaginal discs were dissected from wandering third-instar larvae, fixed, and immunostained using anti-β-gal (Promega; Z378), anti-CBFA2T3 (Abcam; ab33072), and DAPI (Invitrogen; D3571) as previously described by Carroll et al. (2012). To assess the phenotypic effects of *CBFA2T3-GLIS2* and *GLIS2*, UAS transgenes were expressed under control of the epithelial driver *C765-Gal4*, and progeny was observed. Pharate adults were dissected from pupal casings and imaged.

ACCESSION NUMBERS

The sequence data and SNP microarray data have been deposited in the dbGaP database (http://www.ncbi.nlm.nih.gov/gap) under the accession number phs000413.v1.p1. Affymetrix gene expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under GSE35203.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.10.007.

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LETTERS TO THE EDITOR

Novel splicing-factor mutations in juvenile myelomonocytic leukemia

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Myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are heterogeneous groups of chronic myeloid neoplasms characterized by clonal hematopoiesis, varying degrees of cytopenia or myeloproliferative features with evidence of myelodysplasia and a propensity to acute myeloid leukemia (AML).[1] In recent years, a number of novel gene mutations, involving *TET2, ASXL1, DNMT3A, EZH2, IDH1/2,* and *c-CBL,* have been identified in adult cases of chronic myeloid neoplasms, which have contributed to our understanding of disease pathogenesis.[2–7] However, these mutations are rare in pediatric cases, with the exception of germline or somatic *c-CBL* mutations found in 10–15% of chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML),[8] highlighting the distinct pathogenesis of adult and pediatric neoplasms.[9]

Recently, we reported high frequencies of mutations, involving the RNA splicing machinery, that are largely specific to myeloid neoplasms, showing evidence of myeloid dysplasia in adult.[10] Affecting a total of eight components of the RNA splicing machinery (U2AF35, U2AF65, SF3A1, SF3B1, SRSF2, ZRSR2, SF1 and PRPF40B) commonly involved in the 3′ splice-site (3′SS) recognition, these pathway mutations are now implicated in the pathogenesis of myelodysplasia.[10] To investigate the role of the splicing-pathway mutations in the pathogenesis of pediatric myeloid malignancies, we have examined 165 pediatric cases with AML, MDS, chronic myeloid leukemia (CML) and JMML for mutations in the four major splicing factors, *U2AF35, ZRSR2, SRSF2,* and *SF3B1,* commonly mutated in adult cases.

Bone marrow or peripheral blood tumor specimens were obtained from 165 pediatric patients with various myeloid malignancies, including *de novo* AML ($n=93$), MDS ($n=28$), CML ($n=17$) and JMML ($n=27$), and the genomic DNA (gDNA) was subjected to mutation analysis (Supplementary Table 1). The status of the RAS pathway mutations for the current JMML series has been reported previously (Supplementary Table 2).[11,12] Nineteen leukemia cell lines derived from AML (YNH-1, ML-1, KASUMI-3, KG-1, HL60, inv-3, SN-1, NB4 and HEL), acute monocytic leukemia (THP-1, SCC-3, J-111, CTS, P31/FUJ, MOLM-13, IMS/MI and KOCL-48) and acute megakaryoblastic leukemia (CMS and CMY) were also analyzed for mutations. Peripheral blood gDNA from 60 healthy adult volunteers was used as controls. Informed consent was obtained from the patients and/or their parents and from the healthy volunteers. We previously showed that for *U2AF35, SRSF2* and *SF3B1,* most of the mutations in adult cases were observed in exons 2 and 7, exon 1, and exons 14 and 15, respectively.[10] Therefore, we confirmed mutation screening to these 'hot-spot' exons. In contrast, all the coding exons were examined for *ZRSR2,* because no mutational hot spots have been detected. Briefly, the relevant exons were amplified using PCR and mutations were examined by Sanger sequencing, as previously described.[10] The Fisher's exact test was used to evaluate the statistical significance of frequencies of mutations for *U2AF35, SF3B1, ZRSR2* or *SRSF2* in adult cases and pediatric cases. This study was approved by the Ethics Committee of the University of Tokyo (Approval number 948-7).

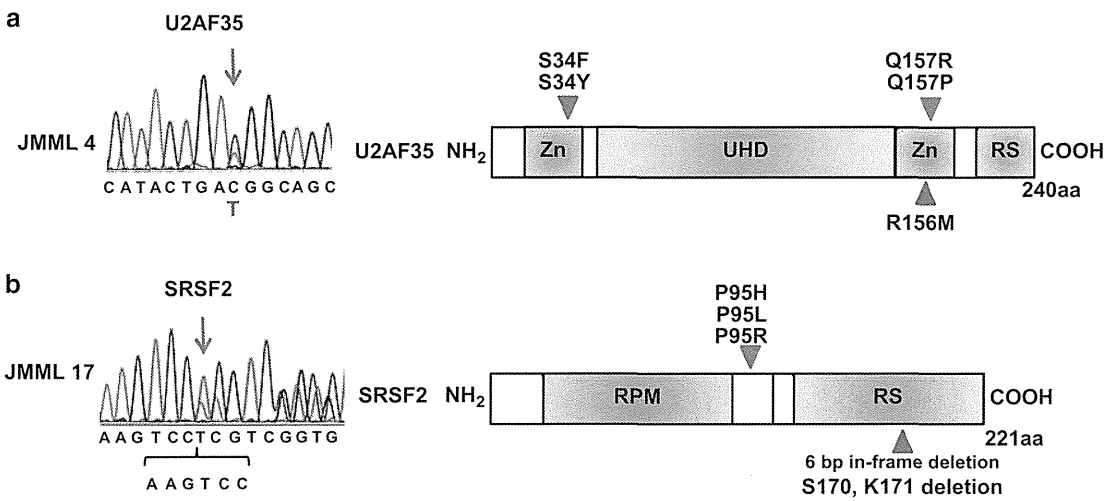

Figure 1. Novel *U2AF35*and *SRSF2* mutations detected in JMML cases. (**a**) Left panel: sequence chromatogram of a heterozygous mutation at R156 in N-terminal zinc-finger motifs of *U2AF35* detected in a JMML case (JMML 4) is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of U2AF35. Red arrow heads indicate hot-spot mutations at S34 and Q157 detected in the adult cases.[10] Blue arrow head indicates the missense mutation at R156. (**b**) Left panel: sequence chromatogram of a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* detected in JMML 17 is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of SRSF2. Red arrow head indicates hot-spot mutation at P95 frequently detected in the adult cases.[10] Blue arrow head indicates a 6-bp in-frame deletion leading to deletion of S170 and K171.

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No mutations were identified in the 28 cases with pediatric MDS, which included 13 cases with refractory anemia with excess blasts, 5 with refractory cytopenia of childhood, 2 with Down syndrome-related MDS, 2 with Fanconi anemia-related MDS, 2 with secondary MDS and 4 with unclassified MDS. Similarly, no mutations were detected in 93 cases with *de novo* AML or in 17 with CML, as well as 19 leukemia-derived cell lines. Our previous study in adult patients showed the frequency of mutations in *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* to be 60/155 cases with MDS without increased ring sideroblasts and 8/151 *de novo* AML patients, emphasizing the rarity of these mutations in pediatric MDS ($P<5.0\times10^{-6}$) and AML ($P<0.02$) compared with adult cases. We found mutations in two JMML cases, JMML 4 and JMML 17. JMML 4 carried a heterozygous *U2AF35* mutation (R156M), whereas JMML 17 had a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* that resulted in deletion of amino acids S170 and K171 (Figure 1). Both nucleotide changes found in *U2AF35* and *SRSF2* were neither identified in the 60 healthy volunteers nor registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) or in the 1000 genomes project, indicating that they represent novel spliceosome mutations in pediatric cases.

U2AF35 is the small subunit of the U2 auxiliary factor (U2AF), which binds an AG dinucleotide at the 3′SS, and has an essential role in RNA splicing.[13] With the exception of a single A26V mutation found in a case of refractory cytopenia with multilinage dysplasia, all the U2AF35 mutations reported in adult myeloid malignancies involved one of the two hot spots within the two zinc-finger domains, S34 and Q157, which are highly conserved across species, suggesting the gain-of-function mutations.[10] In JMML 4, the R156M U2AF35 mutation affects a conserved amino acid adjacent to Q157, suggesting it may also be a gain-of-function mutation, leading to aberrant pre-mRNA splicing possibly in a dominant fashion.

SRSF2, better known as SC35, is a member of the serine/arginine-rich (SR) family of proteins.[14] SRSF2 binds to a splicing-enhancer element in pre-mRNA and has a crucial role not only in constitutive and alternative pre-mRNA splicing but also in transcription elongation and genomic stability.[14] All mutations thus far identified in adult cases exclusively involved P95 within the intervening sequence between the N-terminal RNA-binding domain and the C-terminal RS domain.[10] This region interacts with other SR proteins, again suggesting that the P95 mutation may result in gain-of-function.[10] This proline residue is thought to determine the relative orientation of the two flanking domains of SRSF2, and a substitution at this position could compromise critical interactions with other splicing factors necessary for RNA splicing to take place. In contrast, the newly identified 6-bp in-frame deletion in JMLL 17 results in two conserved amino acids, S170 and K171, within the RS domain. Although it may affect protein–protein interactions, the functional significance of this deletion remains elusive.

JMML is a unique form of pediatric MDS/MPN characterized by activation of the RAS/mitogen-activated protein kinase signaling pathway; in 90% of cases, there are germ line and/or somatic mutations of *NF1*, *NRAS*, *KRAS*, *PTPN11* and *CBL*.[8] Although JMML shares some clinical and molecular features with CMML, its spectrum of gene mutations suggests that it is a neoplasm distinct from CMML.[15] This was also confirmed by the current results that the splicing-pathway mutations are rare in JMML, whereas they are extremely frequent (~60%) in CMML.[10] Although the two JMML cases carrying the splicing-pathway mutations had no known RAS-pathway mutations, both the pathway mutations frequently coexisted in CMML.[8]

To summarize, no mutations of *SF3B1*, *U2AF35*, *ZRSR2* or *SRSF2* are found in pediatric MDS and AML. In our study, except for *ZRSR2*, mutations were examined focusing on the reported hot spots in adult studies, raising a possibility that we may have missed some mutations occurring in other regions. However, these hot spots represent evolutionally conserved amino acids and have functional relevance, it is unlikely that the distribution of hot spots in children significantly differs from adult cases and as such, we could safely conclude that mutations of *SF3B1*, *U2AF35*, *ZRSR2* and *SRSF2* are rare in myeloid neoplasms in children. Finally, mutations of *U2AF35* and *SRSF2* may have some role in the pathogenesis of JMML, although further evaluations are required.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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J Takita[1,2], K Yoshida[3], M Sanada[3], R Nishimura[1], J Okubo[1], A Motomura[1], M Hiwatari[1], K Oki[1], T Igarashi[1], Y Hayashi[4] and S Ogawa[3]

[1]*Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2]*Department of Cell Therapy and Transplantation Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[3]*Cancer Genomics Project, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan and*
[4]*Gunma Children's Medical Center, Gunma, Japan*
E-mail: sogawa-tky@umin.ac.jp

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Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia

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Mutations affecting epigenetic regulators have long been known to have a crucial role in cancer and, in particular, hematological malignancies.[1,2] One of the earliest epigenetic factors described altered in leukemia was the mixed lineage leukemia (*MLL*) protein which is found translocated in 10% of adult acute myeloid leukemia (AML), 30% of secondary AML and >75% of infants with both AML and acute lymphocytic leukemia (ALL). *MLL* is a SET domain-containing protein, which is recruited to many promoters and mediates histone 3 lysine 4 (H3K4) methyltransferase activity, thought to promote gene expression.[3]

In addition to *MLL* fusions, recently, somatic mutations of *UTX* (also known as *KDM6A*), encoding an H3K27 demethylase, were described in multiple hematological malignancies, including multiple myeloma and many types of leukemia cell lines.[4,5] H3K27 methylation is generally thought to cause gene repression. Complimentary to *UTX,* mutations of *EZH2*, a H3K27 methyltransferase, have been reported in both lymphoid and myeloid tumors (Figure 1).[6,7] These mutations lead to altered EZH2 activity and influence H3K27 in tumor cells. Mutations in EZH2, EED and SUZ12, which all cooperate in Polycomb repressive complex 2 have been recently described in early T-cell precursor ALL.[8] Similarly, point mutations affecting the functional jumonji C (jmjC) domain of UTX inactivates its H3K27 demethylase activity. In addition, UTX associates with MLL2 in a multiprotein complex, which promotes H3K4 methylation, and recently *MLL2* has also been found mutated in cancer, further pointing to a common and complex epigenetic deregulation in cancer.[9] In line with the growing evidence for epigenetic regulators as important in tumorigenesis, additional mutations affecting epigenetic regulators such as SETD2, a H3K36 methyltransferase, KDM3B, a H3K9 demethylase, and KDM5C, a H3K4 demethylase, have been reported and are associated with distinct gene expression patterns (Figure 1).[4]

Though the clinical significance of these findings remains to be explored, it is evident that epigenetic deregulation is having an important role in both lymphoid and myeloid leukemogenesis. Furthermore, with novel drugs at hand, such as histone deacetylase inhibitors or demethylating agents that can target and reverse epigenetic alterations, understanding the underlying molecular aberrations is of growing interest.[10] We therefore undertook an effort to examine the prevalence of somatic mutations in genes encoding histone-modifying proteins, in particular, *KDM3B*, *KDM5C*, *UTX*, *MLL2*, *EZH2* and *SETD2*, which previously were reported mutated in cancer.[4,5]

For an initial screen, we analyzed banked diagnostic primary leukemia samples from 44 childhood B-cell ALL and 50 adult AML patients, and, where available, used bone marrow samples obtained in complete remission to validate the somatic nature of the mutations. Samples had been collected with patient/parental informed consent from patients enrolled on Dana–Farber Cancer Institute protocols for childhood ALL (DFCI 00-001 (NCT00165178), DFCI 05-001 (NCT00400946)) or AML treatment protocols of the German-Austrian AML Study Group (AMLSG) for younger adults (AMLSG-HD98A (NCT00146120), AMLSG 07-04 (NCT00151242)), and the study was approved by the IRB of the participating centers.

Using conventional Sanger sequencing of primary leukemia sample-derived genomic DNA, we first screened all coding exons in which mutations have been reported previously.[4,5] Initially, we analyzed a total of 36 of 174 exons (*KDM3B* (2/24), *KDM5C* (9/26), *UTX* (7/29), *MLL2* (8/54), *EZH2* (1/20) and *SETD2* (9/21)) and found 7 non-synonymous tumor-specific aberrations. In AML, we found one *EZH2* mutation (p.G648E) in a t(8;21)-positive, and two *MLL2* missense mutations (p.R5153Q and p.Y5216S; Table 1) and one

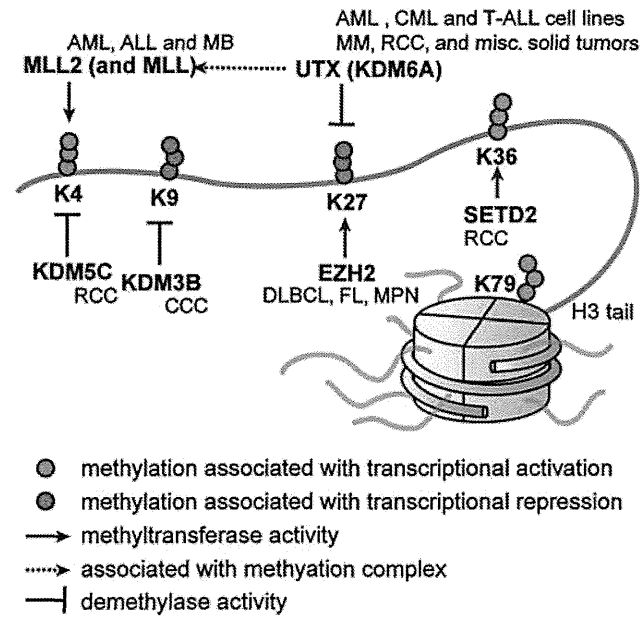

Figure 1. Histone 3 methylation and selected histone demethylases and methyltransferases. Cancers are shown in italics next to the mutated protein they are associated with. MM, multiple myeloma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; RCC, renal cell carcinoma; CCC clear cell carcinoma; MPN, myeloproliferative neoplasm; MB, medulloblastoma.

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BRIEF REPORT

Thrombosis from a Prothrombin Mutation Conveying Antithrombin Resistance

Yuhri Miyawaki, M.Sc., Atsuo Suzuki, M.Sc., Junko Fujita, B.Sc., Asuka Maki, B.Sc., Eriko Okuyama, B.Sc., Moe Murata, B.Sc., Akira Takagi, Ph.D., Takashi Murate, M.D., Ph.D., Shinji Kunishima, Ph.D., Michio Sakai, M.D., Kohji Okamoto, M.D., Ph.D., Tadashi Matsushita, M.D., Ph.D., Tomoki Naoe, M.D., Ph.D., Hidehiko Saito, M.D., Ph.D., and Tetsuhito Kojima, M.D., Ph.D.

SUMMARY

We identified a novel mechanism of hereditary thrombosis associated with antithrombin 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.

From the Departments of Pathophysiological Laboratory Sciences (Y.M., A.S., J.F., A.M., E.O., M.M., A.T., T. Murate, T.K.) and Hematology–Oncology (T.N.), Nagoya University Graduate School of Medicine, the Department of Medical Technology, Nagoya University School of Health Sciences (A.T., T. Murate, T.K.), the Department of Advanced Diagnosis, Clinical Research Center (S.K.), National Hospital Organization, Nagoya Medical Center (S.K., H.S.), and the Department of Transfusion Medicine, Nagoya University Hospital (T. Matsushita), Nagoya; and the Departments of Pediatrics (M.S.) and Surgery 1 (K.O.), University of Occupational and Environmental Health, Kita-kyushu — all in Japan. Address reprint requests to Dr. Kojima at the Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan, or at kojima@met.nagoya-u.ac.jp.

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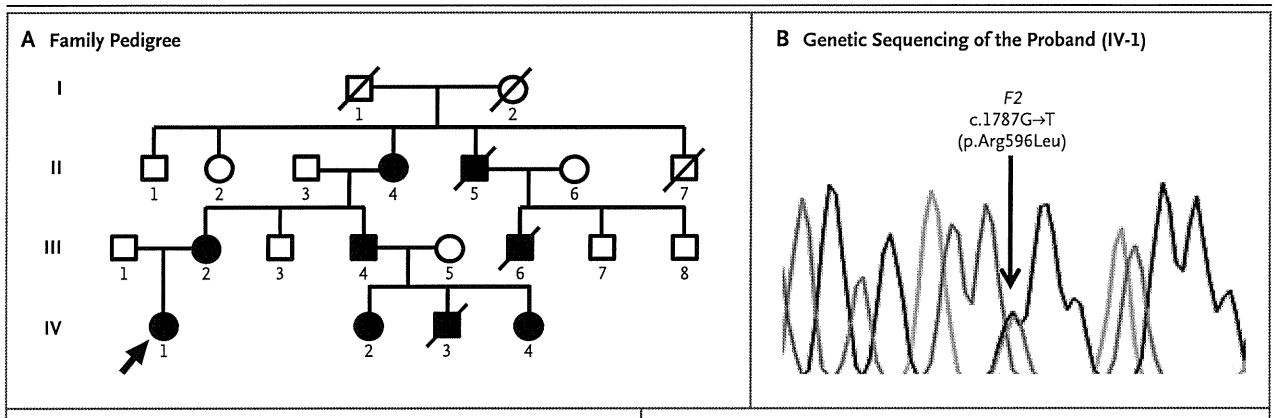
PATIENTS WITH HEREDITARY THROMBOPHILIA OFTEN PRESENT WITH UNusual clinical episodes of venous thrombosis at a young age and recurrence in atypical vessels, often with a family history of the condition.[1] Genetic studies of hereditary thrombophilia have revealed two types of genetic defects: loss-of-function 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]



Allele Frequency

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 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 and various incubation times for antithrombin and recombinant thrombins; the latter are forms of recombinant prothrombins activated by factor Xa.

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™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.

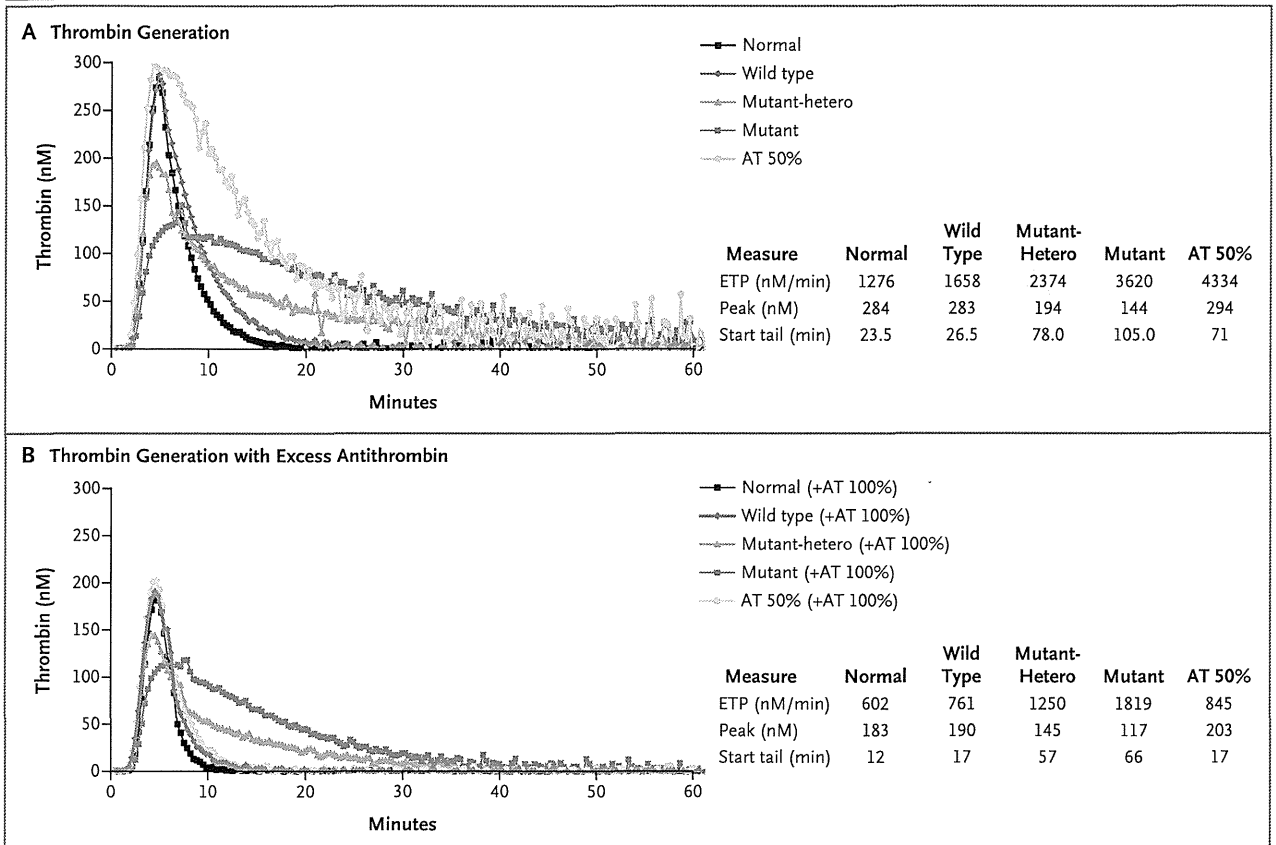
Table 1. Procoagulant and Amidolytic Activities of the Recombinant Prothrombins.*

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 in reconstituted 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.

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



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

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

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.

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 anticoagulant 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 procoagu-

lant 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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Supplementary data

Table of contents

1.	**Table of contents**	P-1
2.	**Suppl. Methods**	P-2
3.	**Suppl. Results**	P-2
4.	**Suppl. Figure Legends**	P-3, 4
5.	**Suppl. Fig. S1. Structural features of the thrombin-antithrombin complex (PDB ID: 1TB6).**	P-5
6.	**Suppl. Fig. S2. Activation of recombinant prothrombins by prothrombinase.**	P-6
7.	**Suppl. Table S1. Primers for PCR amplification of the prothrombin gene.**	P-7
6.	**Suppl. References**	P-8

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 $CaCl_2$ 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 derivatives[2]. 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 thromin-antithrombin complex (PDB ID: 1TB6).

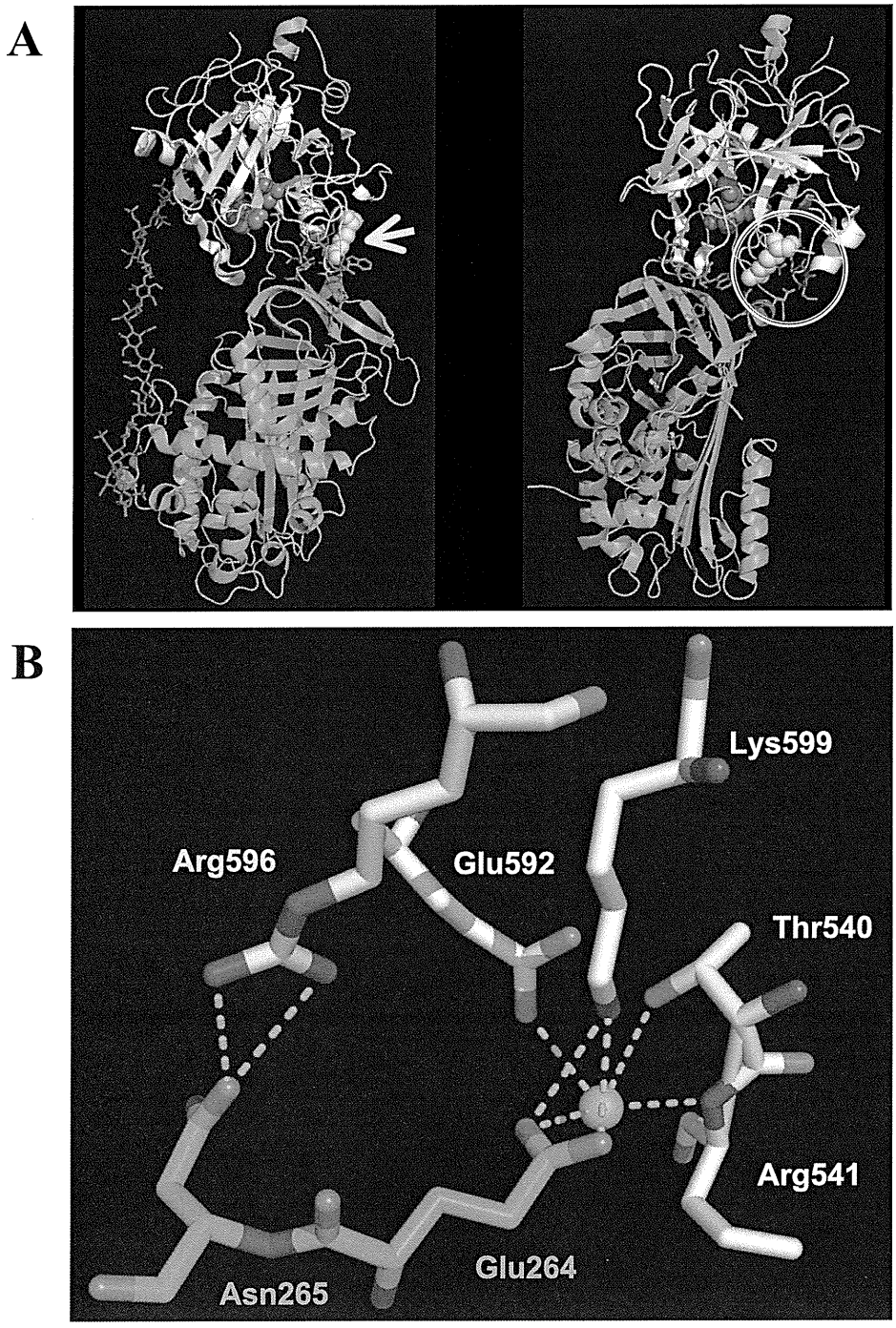
Panel A shows the crystal structure of the thrombin-antithrombin complex with heparin (left) and that of the hidden heparin (right). Thrombin (light blue, light chain; white, heavy chain) and antithrombin (green) are combined via two exosites with heparin (violet stick), the γ-loop binding region, and 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 Lys599[3]. Residues of thrombin and antithrombin are shown in white and green, respectively. The water molecule is shown as a light blue sphere.

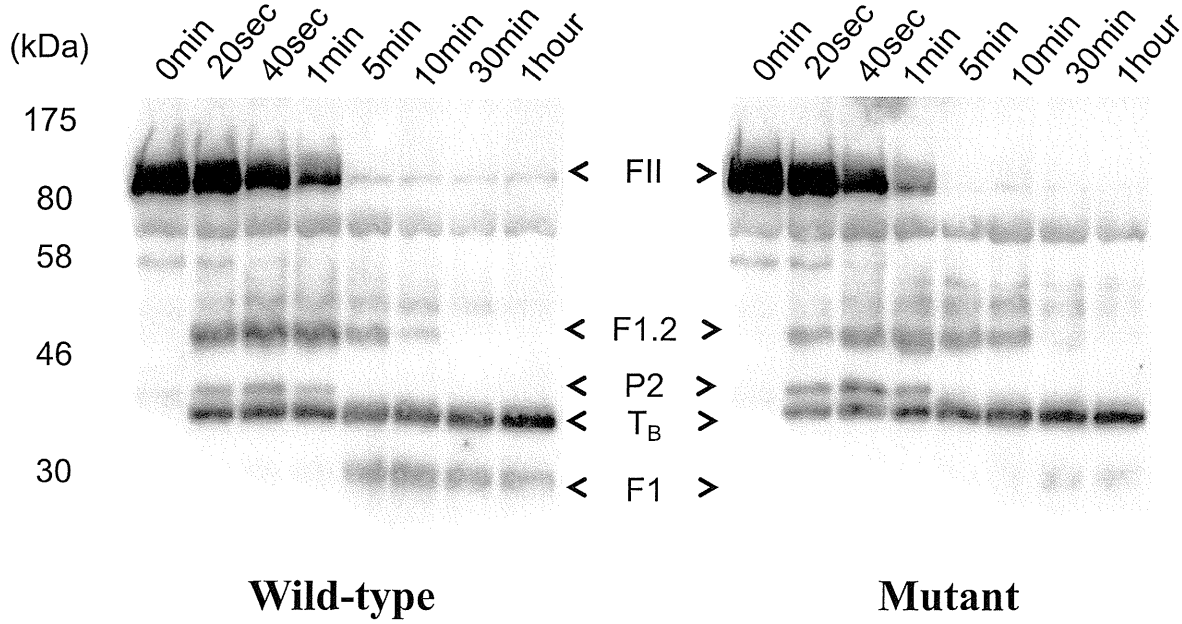
Suppl. Fig. S2. Conversion of recombinant prothrombins by prothrombinase.

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 $CaCl_2$, 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; T_B, B chain of α-thrombin; and F1; fragment 1.

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



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	5'-TGGAGATGGACAGGAGGACT-3'	60	337
	Lw	5'-ACCTACTTAGGGGCCAGCTC-3'		
2	Up	5'-CCTCTCTCAGAAGCCAGCAG-3'	60	388
	Lw	5'-TGAAATGAGGCTGTGAGCAG-3'		
3–4	Up	5'-GCGTGACCAGGGTAAAGGAA-3'	60	493
	Lw	5'-AAACCCACCCCTGAGCTCTT-3'		
5–6	Up	5'-TGGGGGATAGACAACTTTGC-3'	60	499
	Lw	5'-TTCTTGGTTCCCATCCCAG-3'		
7	Up	5'-GTCACACAGGCAGAAAGCAG-3'	60	489
	Lw	5'-CAGAAGCGGCTGTTGTTATT-3'		
8–9	Up	5'-GATCTAGGGGATGGGTGAGG-3'	60	461
	Lw	5'-GGGTCCAGCAGCACACCT-3'		
10	Up	5'-GGGTTCTTAGACCTGGGATTG-3'	60	368
	Lw	5'-CATGATCGCTTTGGAGGACT-3'		
11	Up	5'-GCAGGACACACTGTCTCCCAGAC-3'	60	368
	Lw	5'-AAAAGGGAAAGGGGCTCTTGC-3'		
12	Up	5'-CCAGCTCTGGCGTTTTAGAT-3'	60	400
	Lw	5'-TGAGCCACCAAGAGGTTAGG-3'		
13	Up	5'-AAGTGGGGACAGCAAGAATGA-3'	60	309
	Lw	5'-GAGTCAAGTTCAAGGTCACATCAG-3'		
14	Up	5'-AGGGCCTGGTGAACACATCTTC-3'	60	467
	Lw	5'-CCAGGTGGTGGATTCTTAAGTCTTC-3'		