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Antithrombin Aomori: Identification of a Point Mutation Resulting in Arg^{393}-His Substitution

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Summary. **Abnormal antithrombin(AT), designated antithrombin Aomori, is a functionally inactive AT molecule associated with thrombotic disease. Functional analyses of a patient's plasma showed that the antithrombin activity as well as heparin cofactor activity decreased to approximately half of that of control plasma, suggesting that the AT in the patient's plasma contained a variant AT molecule with impaired antithrombin activity. The elution fractions of the patient's plasma from a heparin-Sepharose column showed two major AT peaks. The first peak with obvious antithrombin activity was eluted at a position similar to the control plasma. The second peak with impaired antithrombin activity was eluted at a higher concentration of sodium chloride, indicating that the variant AT has an increased affinity for heparin. Genomic DNA from peripheral white blood cells was prepared for polymerase chain reaction (PCR), and amplified PCR fragments were sequenced. A codon substitution of CGT(Arg^{393})-CAT(His) was observed in the exon 6 of the AT gene, demonstrating a heterozygous Type II AT deficiency with P1 reactive site variant. This patient represents the first case of a reactive site variant of AT in a Japanese family.**

Key words—antithrombin, reactive site, heparin binding site, thrombosis, polymerase chain reaction.

INTRODUCTION

Antithrombin (AT) is a single chain glycoprotein of molecular weight 58,200, whose structure has been predicted from protein and cDNA sequencing.[1-4] It is a member of the serine protease inhibitors (SERPINs) superfamily,[5] and is the major plasma inhibitor of thrombin, factor Xa and other serine proteases.[6] Quantitative and/or qualitative abnormalities of AT are known to be associated with an increased risk of thromboembolic disorders.[7,8] Conventionally, the disease is classified into two categories[9] based on antigen concentration, progressive antithrombin activity, heparin cofactor activity and heparin-binding activity determined by crossed immunoelectrophoresis: Type I, low functional and immunological antithrombin III; and Type II, showing the presence of a variant antithrombin III. A certain number of subtypes occur in Type II AT deficiency.

AT consists of two important functional domains, i.e., the heparin binding domain and the reactive site. The gene for AT lies on chromosome 1 (1q22-25) and contains 7 exons distributed over 14 kb.[10] Exon 2 of the AT gene encodes the first 104 amino acids of the mature protein—where the heparin binding domain sequence lies—as well as a part of the 32 amino acid signal peptide. Exon 6 encodes amino acids 375-432, where the sequence of the reactive site responsible for inactivation of the coagulation proteinase lies. In Type II deficiency, point mutations of AT affecting the heparin binding domain and/or the reactive site lead to the production of functionally abnormal proteins.[9] In this study, we analyzed a patient with a Type II AT deficiency by polymerase chain reaction/direct DNA sequencing and elucidated a molecular defect responsible for this abnormality.

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MATERIALS AND METHODS

Materials

Sea-Kem HE Agarose was obtained from FMC Co. (Rockland, ME, U.S.A.). Standard heparin was from Sigma (St. Louis, MO, U.S.A.). Rabbit anti-human antithrombin III antibody was obtained from Behringwerke (Marburg, Germany). The chromogenic substrate for thrombin, S-2238, was obtained from Kabi (Sweden). Taq DNA polymerase was from Perkin Elmer Cetus (Norwalk, CT, U.S.A.) and Sequenase ver. 2 from United States Biochemical (Cleveland, OH, U.S.A.). [^{35}S]dATP was purchased from New England Nuclear (Boston, MA, U.S.A.). All other reagents used were as pure as possible.

Assays for progressive antithrombin and heparin cofactor activities

Antithrombin activities of the patient's plasma were determined using standard chromogenic assays[11,12] with some modifications. Briefly, 100 μl of citrated plasma from the patient or control subject was diluted with 7 ml of dilution buffer (0.05 M Tris-HCl, pH 8.4, 0.15 M NaCl). To 450 μl of the diluted sample was added 100μl of 10 NIH U/ml thrombin. After incubation at 37°C for 10, 20, 30 and 45 min, the remaining thrombin activity was measured using synthetic chromogenic substrate S-2238 (progressive antithrombin activity assay). For the measurement of heparin cofactor activity, dilution buffer containing 2.4 U/ml of standard heparin was used instead of the buffer without heparin.

Evaluation of the affinity for heparin

Crossed immunoelectrophoresis (CIE) was performed to assess the heparin binding capacity of AT using 1% agarose containing 25 U/ml of standard heparin in the first dimension and 1.5% of rabbit polyclonal antibody to human ATIII in the second dimension.[13] Ten microliters of each sample was applied into a well and electrophoresis was carried out in the first dimension gel. The gel was cut into slabs and transferred to a glass plate coated with antibody-containing agarose gel. The second electrophoresis was carried out into the second dimension gel at a right angle to the previous direction.

Heparin-Sepharose column chromatography was also performed to assess the affinity of AT for heparin[14]: 0.5 ml of the citrated plasma from the patient or control subject was mixed with the same volume of Tris-buffer (0.05 M Tris-HCl, 0.01 M citrate, pH 7.4), and loaded onto the column (0.9×8 cm) equilibrated with the Tris-buffer containing 0.15 M NaCl. After washing the column with the Tris-buffer containing 0.5 M NaCl, ATs were eluted with a linear NaCl gradient to 1.5 M, starting with 80 ml of each buffer. The concentration of AT in each fraction was estimated by immunoreactivity with rabbit anti-human ATIII antibody. An aliquot from each fraction was spotted onto a nitrocellulose membrane by vacuum aspiration, and the membrane was reacted with the anti-ATIII antibody. The bound antibodies were detected using an ^{125}I-goat anti-rabbit IgG, and the concentration of AT was estimated based on the intensity of the signal.

Polymerase chain reaction

Genomic DNA was prepared from peripheral white blood cells according to a standard procedure.[15] Each exon of AT gene was amplified via a polymerase chain reaction (PCR)[16] using this genomic DNA. Briefly, five-hundred nanograms of the DNA were amplified with 2.5 U of Taq polymerase in a total volume of 100 μl containing 50 pmol of each oligonucleotide primer. The -5′ ends of the reverse primers employed for PCR were labeled with biotin to facilitate purification of the amplified fragment.

DNA sequencing

The amplified fragment was purified with Dynabeads M-280 streptavidin (Dynal) according to the manufacturer's instruction. Single strand DNAs from both strands were obtained after alkaline denaturation. DNA sequence was performed on both strands with Sequenase ver. 2 by the dideoxy-mediated chain-termination method.[17]

RESULTS

Patient profile

The propositus was a 55-year-old male who was healthy until February, 1990, when he was hospitalized because of severe headaches and visual disorder. He was diagnosed as having cerebral sinus thrombosis based on the findings of the cerebral angiography. Laboratory examinations were almost normal, except for a low antithrombin (AT) acivity (52.8%) with normal levels of AT antigen (28.0 mg/ml), suggesting that the patient had a heterozygous variant of AT. The pedigree of his family and their heparin

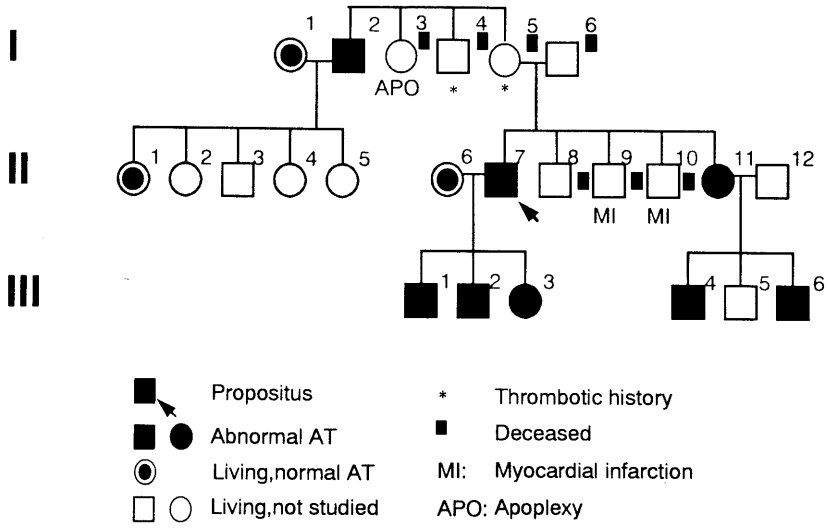

Fig. 1. Pedigree of the patient's family. The propositus is indicated by an arrow. Roman numerals refer to generation and arabic numbers refer to individuals of each generation.

cofator activities are shown in Fig. 1 and Table 1. Eight out of 11 family members of three generations showed decreased heparin cofator activities (normal range; 75-125%) with normal plasma concentrations of AT (25.4±7.0 mg/ml), indicating an autosomal dominant trait of inheritance.

Functional analyses of the propositus' plasma

Fig. 2 shows the anti-thrombin activity of the plasma in the absence or presence of heparin. After incubation with normal plasma, thrombin was gradually inactivated by AT, reaching 90% inhibition at 30 min of incubation. In the presence of heparin, thrombin inactivation by normal plasma was greatly enhanced and more than 90% inhibition was observed at 3 min of incubation. In contrast, the propositus' plasma showed a decreased inhibition of thrombin both in the absence or presence of heparin. The progressive antithrombin activity of the plasma was approximately half of normal plasma. Heparin cofator activity of the plasma was about three-fourths of normal plasma. Since approximately 25% of heparin cofactor activity in the plasma is attributed to heparin cofactor II,[18] these results indicate that AT in the patient's plasma contained a variant without thrombin inhibitory activity.

Heparin-binding capacity of AT was first assessed by CIE: the electrophoretic pattern of the patient's plasma was similar to that of normal plasma (data not shown), indicating that the heparin binding domain of AT was retained. Next, the heparin-Sepharose column chromatography was carried out, and both antithrombin activity and AT antigen levels in the fractions were measured (Fig. 3). A single AT peak was observed in the control plasma (Fig. 3A), but two distinct AT peaks were observed in the patient's plasma (Fig. 3B). The first peak, which shows evident heparin cofactor activity, was eluted at a similar position of the control plasma, whereas the second peak was eluted at the higher concentra-

Table 1. Functional assays of antithrombin in the AT Aomori family

Subject	Sex	Age	Heparin cofactor activity (%)	AT antigen level (mg/dl)
I-2	M	77	67.0	22.5
II-7*	M	55	52.8	28.0
III-1	M	26	59.0	26.6
III-2	M	24	60.0	22.8
III-3	F	20	65.0	23.9
III-4	M	26	60.0	27.3
III-6	M	19	65.0	23.0

*Propositus: Heparin cofactor activity was measured with an automated coagulomaster (COBAS-FARA II) and AT antigen level was measured by single-radial immunodiffusion method using a commercial kit (Behringwerke).

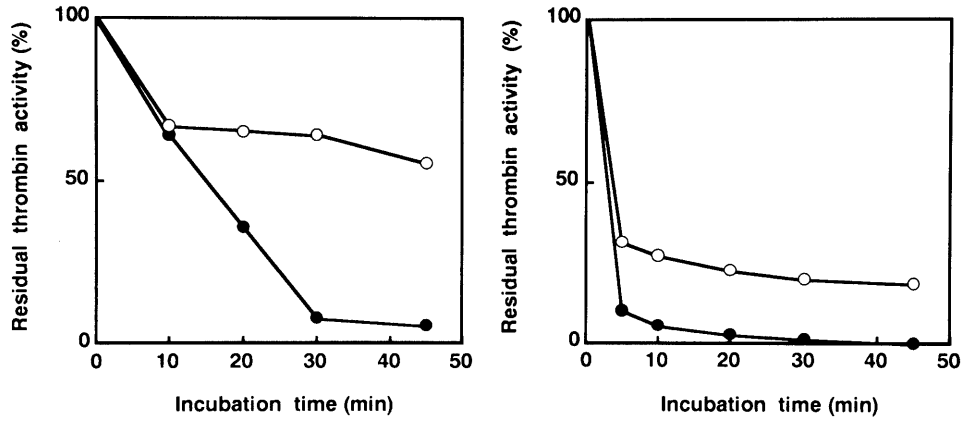

Fig. 2. Inactivation of thrombin by normal and propositus' plasma. Diluted plasma was incubated with thrombin in the absence or presence of heparin for a designated time, and the residual thrombin activity was determined using chromogenic substrate S2238. The left figure shows the progressive antithrombin activity and the right figure shows the heparin cofactor activity of normal (●—●) and the propositus' plasma (○—○).

tion of NaCl (≈ 0.89 M) and shows the discrepancy between antigen and activity. Taken together with the previous results, it clearly demonstrates that the patient's plasma contains variant AT molecule(s) with increased heparin affinity but impaired antithrombin activity.

DNA sequencing of the propositus' antithrombin gene

DNA fragments of the AT gene were obtained by polymerase chain reaction. Primers employed for amplification of the exon 6 of the AT gene were as follows: forward primer, 5′-CTGCAGGTAAATG-AAGAAGGC; reverse primer, 5′-AGGAAGAGGT-GCAAAGAATAAG. The amplified fragments with 211 bp in size were purified as described in Materials and Methods section, and then sequenced. Fig. 4 shows the results of the DNA sequence of both strands. In the left figure an additional band "A" as well as "G" is seen at the position of the second base of Arg^{393} codon, indicating that the patient is heterozygous for this sequence. This point mutation is further verified by the sequence analysis of a non-coding strand showing both "C" and "T" bands at the same position. This G-A codon substitution resulted in the replacement of Arg^{393} with His. Furthermore, other exons of the AT gene were also amplified and sequenced. There was no additional mutation (data not shown), so this G-A substitution was responsible for this abnormality.

DISCUSSION

Since Sas et al. reported a family with thrombophilia caused by abnormal antithrombin (AT), now known as antithrombin III 'Budapest',[19,20] more than 30 types of molecular defects responsible for Type II AT deficiencies has been identified.[9] The patient presented here was diagnosed as Type II AT deficiency based on functional assays, and represents the first case of the variant AT with an impaired reactive site described in a Japanese family.[21] DNA sequencing of the AT gene revealed that the molecular defect responsible for this abnormality was CGT-CAT codon substitution at amino acid residue 393. This CG-CA transition is thought to be a hotspot for point mutation; in fact, more than 50% of the AT mutations so far described have been caused by CG mutation.[22]

Arg^{393} of the AT molecule is located in the P1 site of the reactive site. As previously proposed, amino acid residues within the reactive site of SERPINs play a critical role for the reactivity to protease and protease specificity.[23] So far, several point mutations have been reported within the reactive site of AT that affect the antithrombin activity of AT. The same Arg^{393}-His mutation has been described in four other kindreds: antithrombin III-Glasgow;-Abranches; -Sheffield;-Chicago.[24–27] Since AT is the most important inhibitor of thrombin in plasma, a close relationship between the reactive site mutation and throm-

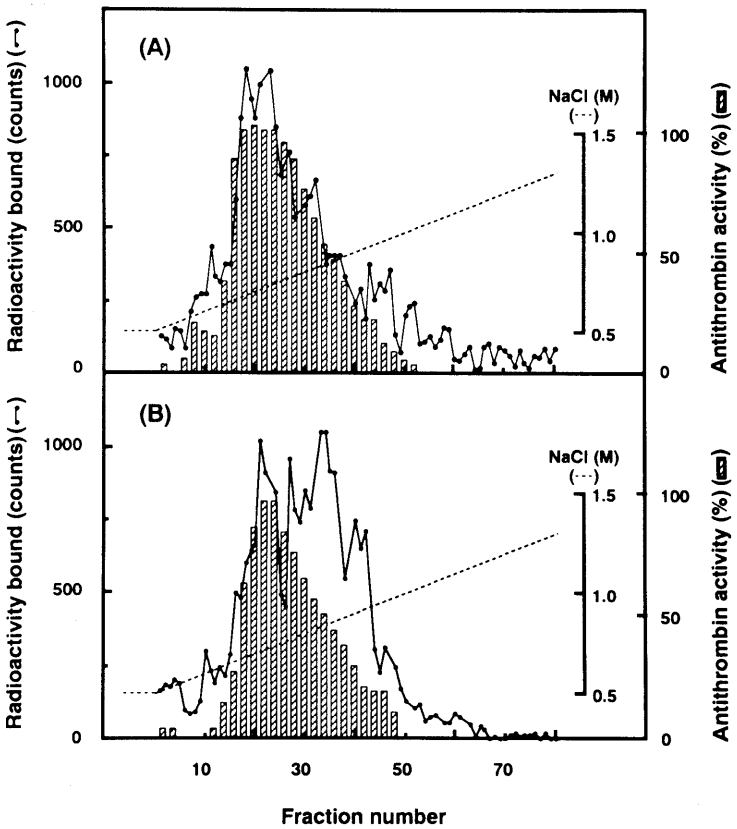

Fig. 3. Elution profile of heparin-Sepharose column chromatography. Fig. **A** shows the elution profile of a control plasma; Fig. **B** shows that of the patient's plasma. The fraction volume was 1.7 ml.

botic diseases has been described (prevalence rate of thromboembolism: 58%).[28] However, in this family only the propositus, who had been suffering from diabetes mellitus, had obvious thrombotic events. His relatives had no apparent thrombotic event as seen in other four kindreds. Several factors affecting blood coagulation/fibrinolysis system (fibrinogen, heparin cofactor II, protein C, protein S, plasminogen and α2-plasmin inhibitor) were measured, but all data were within normal ranges. At present, we cannot explain why this family did not present thrombotic episodes; more detailed investigations of the blood coagulation/fibrinolysis system are necessary to answer the problem.

A similar mutation is also known to show an increased affinity for heparin.[24,26] Although the variant molecule was eluted at somewhat lower concentrations of NaCl as compared to the previously reported case (1.05 mol/l),[14] we clearly demonstrated that the patient's plasma contained the variant AT molecule with high heparin affinity (Fig. 3). Moreover, other exons of the AT gene have been sequenced as well as exon 6, but no additional mutation was found. These results suggest that the increased affinity of this

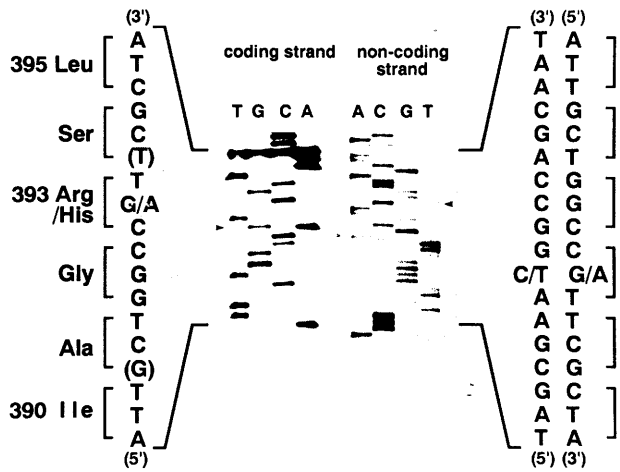

Fig. 4. Sequencing gels showing the AT Aomori allele. Each sequence should read in the 5′-3′ direction. The left figure represents the sequence of the coding strand and the right figure represents a sequence of the non-coding strand. Arrows indicate the position of the second base of the Arg^{393} codon.

variant for heparin is due to this substitution by itself. Previous reports also support this idea. Owen et al. proposed that an ionic bond may be formed between the positively charged Arg^{393} and the adjacent residues to maintain AT in a constrained, non-activated configuration.[29] After the binding of heparin to the heparin binding site, the induced conformational changes of the AT molecule may result in the disruption of this ionic bond on the basis of observations by the fluorescence spectra or circular dichroism.[30,31] Arg^{393}-His substitution results in the loss of this charged residue in P1 variant of AT and may induce a conformational change in the AT molecule, exposing more basic amino acids that can participate in heparin binding. AT Pescara with Arg^{393}-Pro substitution also has an increased affinity for heparin,[29] indicating that P1 variants, which are unable to form this ionic bond, have an increased heparin affinity. Our results are in accord with this explanation.

In the acute period of the cerebral sinus thrombosis of the patient, concentrated antithrombin infusion together with urokinase and glycerol were effective. After the acute phase elapsed, treatment with warfarin was effective for the prevention of thrombosis.[21] We call this abnormal antithrombin "Antithrombin Aomori", based on patient's habitat.

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