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Antithrombin Cambridge, 384 Ala to Pro: a new variant identified using the polymerase chain reaction

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An antithrombin III variant was identified in the plasma of a female patient with a history of recurrent thromboses. The variant was shown to have normal antigenic levels but reduced heparin and progressive inhibitory activity consistent with an abnormality affecting function at the reactive centre. Polymerase chain reaction amplification of exon 6 of the gene with direct sequencing showed a point mutation resulting in the substitution of a proline for alanine at position 384. This substitution will predictably alter the conformation of the peptide loop containing the reactive centre of the molecule.

Antithrombin III; Pathological variant; Polymerase chain reaction; Serpin

1. INTRODUCTION

Human antithrombin III (ATIII) is a single-chain 432 amino acid glycoprotein of molecular weight 58 000. It is a member of the serpin superfamily and is the major inhibitor of a number of serine proteases in the coagulation pathway including thrombin [1,2]. Under normal physiological conditions this inhibition is relatively slow but in the presence of heparin, it is greatly accelerated [3]. Its importance as an inhibitor is emphasised by the recurrent thromboses that occur in individuals with abnormal or deficient ATIII.

We describe here a new variant, with defective protease inhibitory activity but normal heparin binding and in which the underlying genetic mutation was established by means of the polymerase chain reaction (PCR) [4].

2. MATERIALS AND METHODS

2.1. *Materials*

All reagents were obtained from Sigma unless otherwise stated. Deoxynucleotides and Sephadex G100 were obtained from Pharmacia-LKB Biotechnology, *Thermus aquaticus* (*Taq*) polymerase from Perkin Elmer Cetus, and Ultrapure agarose from Gibco-BRL. DNA sequencing used Sequenase version 2.0 (modified T7 DNA polymerase) were obtained from the US Biochem. Corp. Chromogenic substrate Chromozym TH was obtained from Boehringer Mannheim and S2222 from Kabi Diagnostica.

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2.2. *Antithrombin measurements in plasma*

Antithrombin antigen was measured by competitive ELISA. Antithrombin anti-IIa activity was measured in the absence (antithrombin progressive activity) or in the presence of heparin (heparin cofactor activity) according to Abildgaard et al. [5] using the chromogenic substrate Chromozym TH. Anti-Xa activity was measured according to [6] in the presence of heparin (anti-Xa heparin cofactor) using the substrate S2222. All values were expressed as a percentage of a reference plasma defined as 100%. Crossed immunoelectrophoresis was performed according to [7] with or without the addition of heparin (25 U/ml) in the first dimension. Heparin affinity chromatography of patient plasma was performed using a heparin sepharose column (7.5 × 1 cm) and a 0.15–1.5 M NaCl gradient to elute ATIII [8].

2.3. *Amplification of exon 6 of the ATIII gene*

Oligonucleotides (CTGCAGGTAAATGAAGAAGGCAGT GA) and 5′(TTACTTCTGTTCACAAACCAAAAATA) spanning positions 1202–1450 within exon 6 were synthesised on an Applied Biosystems DNA synthesizer and used as amplification primers without further purification. PCRs were performed in 100 μl reaction volumes containing 1 μg of genomic DNA, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 100 pmol

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of each of the amplification oligonucleotides, in 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, and gelatin at 200 μg/ml. Samples were heated to 100°C for 5 min to denature the DNA, briefly spun and placed in a heating block at 55°C for 2 min. 2 U *Thermus aquaticus* (*Taq*) polymerase was added to each sample and the tubes overlaid with 100 μl light mineral oil. Samples were subjected to 35 cycles of amplification consisting of a 20-s extension step at 76°C, a 1-s 94°C denaturation step, and a 1-s 55°C annealing step. On the final cycle the extension time was increased to 10 min. After the amplifications were completed, 18 μl of each reaction was analyzed on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml).

Prior to sequencing, the amplified DNA was purified on a Sephadex G100 column [9].

2.4. *Direct sequencing of the PCR product*

A sequencing oligonucleotide 5'(AGAATAAGAACATTTT ACTT) spanning bases 1390–1409 of the non-coding strand of exon 6 was synthesised as described for the amplification primers. Sequencing primers were purified before use on 20% denaturing polyacrylamide gels. Sequencing was otherwise as reported previously [10].

3. RESULTS

3.1. *Case history*

The propositus was a 30-year-old woman who, at the age of 17 years, developed a deep vein thrombosis (DVT) 2 months after commencing the oral contraceptive pill. She suffered a second spontaneous DVT at age 21 and a third during her first pregnancy at age 29. Her mother also had recurrent spontaneous venous thromboses from the age of 17 years.

3.2. *Antithrombin measurement in plasma*

Plasma antithrombin assays (table 1) showed both the propositus and her mother to have abnormal heparin cofactor and progressive activity but normal antigenic levels. Crossed immunoelectrophoresis demonstrated normal heparin affinity. These findings are consistent with an abnormality primarily affecting inhibitory function at the reactive centre.

Table 1

Results of plasma antithrombin assays

	Antigen	Inhibitory activity (n = 100%)		
		With heparin		Without heparin
		IIa	Xa	IIa
Propositus	95	54	46	63
Mother	98	58	50	67
Brother	90	102		
Sister	95	105		

3.3. *Heparin sepharose chromatography*

ATIII from patient plasma behaved similarly to normal ATIII on heparin sepharose and was eluted as a single peak with 0.75 M NaCl.

3.4. *Amplification and sequencing of exon 6*

Amplification of exon 6 resulted in a single fragment of 249 base pairs (bp) (fig.1). Sequencing of this amplified DNA revealed two bands at nucleotide position 1246 (fig.2) representing the normal guanine (G) and the mutant cytosine (C) bases. The derived sequences from the anti-sense strand are, therefore, GCA and CCA coding for alanine and proline, respectively.

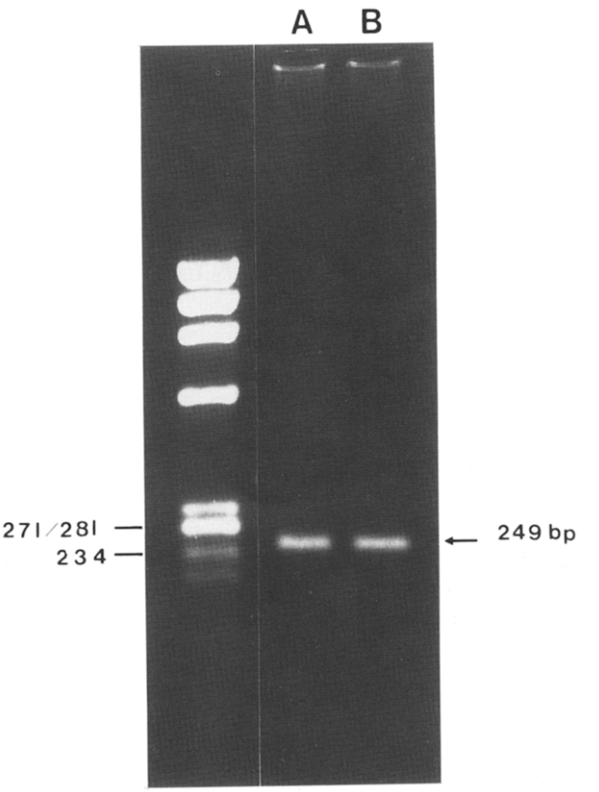

Fig.1. Analysis of the amplified DNA on a 1.5% agarose gel in 1 × Tris-borate/EDTA buffer (TBE) containing ethidium bromide (0.5 μg/ml). The left-hand lane contains a size marker, 3.2 μg φX174/HaeIII, lane A 18 μl amplified ATIII Cambridge, and lane B 18 μl amplified control DNA.

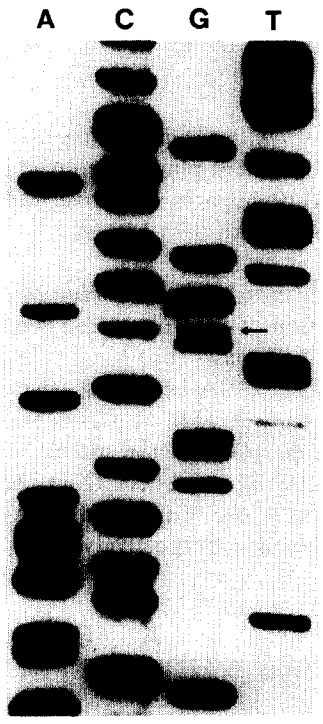

Fig.2. Direct sequence analysis of the antisense strand of ATIII Cambridge indicating a mutation in the gene at codon 384. Reading from the bottom of the gel, the sequences are complementary to the sense strand. Thus, in the normal antithrombin III gene, codon 384 which codes for alanine is CGT (GCA on sense strand), whilst in antithrombin Cambridge, codon 384 which codes for proline, is GGT (CCA on sense strand).

4. DISCUSSION

We report here a new antithrombin variant, antithrombin Cambridge, that has normal heparin binding but has lost its thrombin inhibitory activity. This variant is due to a single point mutation resulting in the replacement of the normal alanine residue at 384 by a proline. Alanine 384 is separated by 9 residues from the reactive centre of antithrombin at arginine 393. The conformation of this region of the antithrombin molecule is not known though predictions can be made from the crystallographic structure of another serpin, α_1-antitrypsin [11]. This predicts that alanine 384 will lie near the base of an exposed loop of peptide which has the reactive centre at its distal end. A feature of the proposed mechanism for the inhibitory activity of the serpins is that the base of the reactive centre loop (i.e. residues 382–385) can reversibly move in and out of the adjacent large A pleated sheet. This potential movement holds the loop in the stressed conformation presumed necessary for inhibitory activity. Thus, all the inhibitory members of the family have a conserved region, 382–385, consisting of small side-chain amino acids principally alanine, glycine, serine or threonine. Exceptions are the non-inhibitory serpins angiotensinogen and ovalbumin which have bulky residues (glutamic acid, valine) in this region and hence do not have the stressed conformation of other members of the family [12]. The presence of the imino acid proline at position 384 in antithrombin Cambridge will predictably have a similar effect, i.e. a distortion of the base region of the reactive centre loop with a consequent loss of movement of this region and hence of inhibitory activity.

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