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Regular Article

Antithrombin heparin binding site deficiency: A challenging diagnosis of a not so benign thrombophilia

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ABSTRACT

Background: Hereditary antithrombin (AT) deficiency is a rare autosomal dominant disorder characterised by decreased AT activity in plasma and predisposition to recurrent venous thromboembolism (VTE). Thrombotic risk is thought to vary according to the subtype of deficiency, with Heparin Binding Site (HBS) deficiencies being the less thrombogenic.
Objectives and Methods: The study population consisted of 82 genetically confirmed HBS deficient patients sharing six different mutations. Plasma samples of 35 of them, including one homozygous patient, were used for the evaluation of 4 commercial activity assays in their ability to diagnose HBS deficiency. We assessed mutation-specific prevalence of venous and arterial thrombosis and the contribution of additional thrombophilic risk factors.
Results and Conclusions: Only one assay showed 100% sensitivity for all HBS mutations. The other ones failed mainly in the cases with p.Pro73Leu and p.Arg79His mutations. Shortening of incubation time resulted in an increase in sensitivity. In one patient, a novel HBS mutation, p.Asn77His, was identified, a quite exceptional and important finding given the restricted number of causal mutations reported so far in AT HBS deficiency. The overall prevalence of VTE in our study population (35%) was higher than previously reported (6-8%) in these patients. The presence of additional thrombophilic risk factors such as Factor V Leiden or prothrombin gene mutation G20210A contributed to a higher risk of VTE. Interestingly, the p.Pro73Leu and p.Arg79His mutations were associated with a high prevalence of arterial thrombosis. Our data suggest that AT HBS deficiencies are probably more prevalent and less benign than previously assumed.

Introduction

Antithrombin (AT), a plasma serine protease inhibitor, is the major inhibitor of thrombin and other coagulation proteases. Its activity can be accelerated up to 1000-fold by heparin. Hereditary AT deficiency is a rare autosomal dominant disorder characterised by recurrent venous thromboembolism (VTE), with first event often presenting at young age. Inherited AT deficiencies can be divided into two types, quantitative (type I) and qualitative (type II). The latter is subclassified depending on whether the mutation affects binding to heparin (Heparin Binding Site, HBS), the reactive site (Reactive Site, RS) or both (Pleiotropic Effect, PE). In the overall population, the prevalence varies from 1 in 500 to 1 in 5000 and consists mainly of type II deficiencies while in symptomatic patients, type I represents up to 80% of all AT deficiency cases [1,2]. HBS variants are thus thought to be the less thrombogenic. However, if present in homozygous state or associated with other thrombophilic risk factors, such as Factor V (FV) Leiden or prothrombin (PT) G20210A mutation, thrombotic risk is markedly increased [2–4].

Types and subtypes of AT deficiency are defined by plasmatic AT activity and antigen levels in combination with molecular analysis of the AT gene *SERPINC1*. The test of choice for diagnosis of AT deficiency is the activity measurement. Current methods quantify AT activity by chromogenic tests measuring inhibition of thrombin [Factor IIa (FIIa)] or Factor Xa (FXa) by AT in the presence of heparin. Despite the numerous functional assays available on the market, no single one appears to be able to detect all defects. Thrombin-based assays have the potential problem that the added thrombin is inhibited by heparin cofactor II (HC-II). This effect can lead to an overestimation of the AT concentration by 5-10% [5]. Although not relevant in individuals with normal AT levels, it is clinically important in diagnosing AT deficiency. Bovine thrombin is not, or only minimally, inhibited by HC-II and today, most commercial FIIa-based assays use bovine instead of human thrombin. On the other hand, mutations in the reactive site affecting binding with thrombin more than FXa may go undetected with FXa-based tests. The AT Cambridge II mutation (p.Ala416Ser) is such an example [6,7].

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Our aim was to study the performance of four commercial AT activity assays, including the three most commonly used in Belgium, in the identification of AT HBS variants. We tried to ameliorate the detection by adjusting incubation time. We report the clinical presentation of HBS deficiencies in our study population and investigated the impact of FV Leiden and prothrombin G20210A mutation on the prevalence of thrombotic events.

Materials and Methods

Patients

All AT HBS deficient patients genotypically confirmed in our centre between August 1990 and December 2014 were included.

Phenotypic diagnosis of AT deficiency was performed in our laboratory or by the referring centre, determining AT activity with the locally used method.

Clinical and laboratory data were obtained using clinical records and a standardised questionnaire completed by the patient's physician. We collected information on venous and arterial thrombotic events (ATE), age at first presentation, recurrent events, presence of acquired risk factors for VTE or ATE and results of thrombophilia screening (lupus anticoagulant, anticardiolipin antibodies, protein C activity, free protein S antigen, resistance to activated protein C, FV Leiden and PT G20210A mutation). In two patients, results of PT G20210A were not available. This study was approved by the Ethical Committee of the Universitair Ziekenhuis Brussel and informed consent was obtained from patients and controls.

Patient Samples

For the performance study of the commercial assays, plasma samples from 35 patients with HBS deficiency and 20 healthy volunteers were used. Blood was taken distant from or in the absence of acute thrombotic event and heparin treatment was excluded. Citrated plasma was obtained by centrifugation for at least 10 minutes at 1500 g and stored in aliquots at −70 °C.

Genomic DNA was extracted from peripheral blood leukocytes using standard methods.

AT Plasma Assays

Four chromogenic AT activity assays were evaluated. The three most commonly used FXa-based tests in Belgium, i.e. HemosIL® Liquid Antithrombin (LIQ) (Instrumentation Laboratory, Bedford, MA, USA), Chromogenix Coamatic® Antithrombin (COA) (Instrumentation Laboratory, Bedford, MA, USA), Innovance® Antithrombin (INN) (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), and one FIIa-based assay, Biophen® Antithrombin (BIO) (Hyphen Biomed, Neuville-sur-Oise, France), were performed on ACL TOP500 coagulation analyser from Instrumentation Laboratory. The reference interval comprises values from 80% to 120%. The main differences between the assays are the source of substrate, the heparin concentration and the incubation time (Table 1).

Table 1
Standard characteristics of the different commercial AT activity assays performed on ACL TOP500.

	LIQ	COA	INN	BIO
Substrate source	Bovine FXa	Bovine FXa	Human FXa	Bovine FIIa
Incubation time	100-140 s	100-140 s	180-190 s	55-65 s
Heparin concentration	3000U/mL	3000U/mL	1500U/mL	5000U/mL

LIQ = HemosIL ®Liquid AT, COA = Coamatic® AT, INN = Innovance®, BIO = Biophen® AT.

AT antigen levels were measured using Laurell rocket immunoelectrophoresis with a rabbit anti-human AT antibody (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany).

Adaptation of Incubation Time

We tried to optimize the BIO and COA assay conditions by modifying the incubation time, based on literature data [7,8]. We changed the incubation time range from 55-65 s to 25-35 s for the BIO assay and from 100-140 s to 110-111 s for the COA assay.

Molecular Analysis

In index cases, molecular analysis of the antithrombin gene, *SERPINC1*, consisted of sequencing of all 7 exons and intron/exon boundaries. In relatives, only the exon containing the familial mutation was investigated. Sequencing analysis was performed essentially as described previously [9]. Since 2012, coding regions were amplified with newly designed primer pairs harbouring a M13-tag (supplementary table S1) with a touchdown-PCR protocol. After purification, all fragments were sequenced using the BigDye Terminator Cycle sequencing kit (Life Sciences, Guilford, CT, USA) on an ABI 3130XL or ABI 3730 genetic analyser (Life Sciences, Guilford, CT, USA).

Mutations are described according to Human Genome Variation Society guidelines. The cDNA reference sequence used was NM_000488.2.

Statistics

Statistical and graphical analysis was performed with Analyse-it software, version 2.26 (Analyse-it Software Ltd, Leeds, UK) and Microsoft Excel 2010 (Microsoft, Redmond, USA). Differences between patient groups were assessed using Fisher's exact test. P values ≤ 0.05 were considered significant. Patients with missing data were excluded from statistical analysis.

Results

Patient Characteristics

Sixteen families, consisting of the index patient and at least one relative with AT HBS deficiency and 35 individual cases, in total 82 individuals were eligible for this study.

All patients were successfully genotyped and 6 different mutations responsible for AT HBS deficiency (p.Pro73Leu, p.Asn77His, p.Arg79His, p.Arg79Cys, p.Leu131Phe, p.Gln150Pro) were identified. Except for p.Asn77His, all mutations have been reported previously.

Performance of Commercial AT Assays

Plasma samples from 35 of the 82 type II HBS deficient patients and 20 healthy volunteers were analysed with the different assays. Detailed results are shown in Table 2 and graphically represented in Fig. 1. The INN assay identified all patients correctly as pathological, whereas the 2 other anti-FXa assays measured normal AT activity values in some patient samples. The LIQ and the COA assay showed discrepancies for 14/35 and 9/35 patients, respectively. The FIIa-based BIO assay yielded significantly higher activity levels when compared to the FXa-based COA assay with an absolute average increase of 25%. Accordingly, more than 60% of our patients were misclassified as normal when measured with the thrombin-based assay. The performance characteristics of the assays are summarized in Table 3.

One of the 35 samples belonged to a patient homozygous for the p.Leu131Phe mutation. This patient was correctly identified as pathological with all studied assays although a great discrepancy was noted between the activity measured with the INN assay and the other assays.