Clinical Biochemistry 47 (2014) 1337–1340

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Short Communication

Discordant diagnoses obtained by different approaches in antithrombin mutation analysis

Søren Feddersen, Mads Nybo *

Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark

ARTICLE INFO

Article history:
Received 2 May 2014
Received in revised form 10 June 2014
Accepted 11 June 2014
Available online 20 June 2014

Keywords:
Antithrombin
Mutation analysis
Risk assessment

ABSTRACT

Objectives: In hereditary antithrombin (AT) deficiency it is important to determine the underlying mutation since the future risk of thromboembolism varies considerably between mutations. DNA investigations are in general thought of as flawless and irrevocable, but the diagnostic approach can be critical. We therefore investigated mutation results in the AT gene, *SERPINC1*, with two different approaches.

Design and methods: Sixteen patients referred to the Centre for Thrombosis and Haemostasis, Odense University Hospital, with biochemical indications of AT deficiency, but with a negative denaturing high-performance liquid chromatography (DHPLC) mutation screening (routine approach until recently) were included. As an alternative mutation analysis, direct sequencing of all exons and exon–intron boundaries without pre-selection by DHPLC was performed.

Results: Out of sixteen patients with a negative DHPLC mutation screening, discordant results were found in ten patients (62.5%) when using direct sequencing: Eight had the Basel mutation (c.218C>T), while two had the Cambridge II mutation (c.1246G>T). For seven of the ten patients this meant an altered clinical risk-assessment for future thromboses.

Conclusions: Awareness must be drawn to the possibility of differences in DNA diagnostics in general and advances when using newer techniques in particular. One should consider re-analysis of results obtained by earlier sequencing strategies, as clinically important information can be overlooked.

Introduction

It is well-known that hereditary antithrombin (AT) deficiency predisposes to venous and arterial thromboses [1], and examination of AT deficiency is an important part of thrombophilia testing. AT is a member of the serpin superfamily with the physiological function to inhibit thrombin and coagulation factor Xa (and to lesser extent factors IXa, XIa and XIIa) [2]. Hereditary AT deficiency is inherited as an autosomal dominant trait, where most cases are heterozygous and found in 4% of families with inherited thrombophilia, 1% of patients with a first-time diagnosis of deep venous thrombosis (DVT), and in 0.02% of healthy individuals [3]. *SERPINC1*, the gene encoding AT, is localised on chromosome 1q23–25.1 and consists of seven exons spanning 13.4 kb [4]. The most frequent genetic defects responsible for hereditary AT deficiency are missense mutations, but also nonsense mutations, splice-site mutations, deletions and insertions have been reported (please see the Human Gene Mutation Database, www.hgmd.org).

First-line investigation of AT deficiency depends on functional assays, but significant inter-laboratory, inter-individual and intra-individual variability impedes the diagnosis [5]. Also, different types can be identified (type I characterized by low protein levels and type II characterized by reduced activity), where type II AT deficiency caused by defects in the heparin-binding site has been found less thrombogenic [3]. Therefore, genetic testing is important to disclose the exact reason for AT deficiency and to determine a correct prophylactic strategy. Furthermore, detection of a mutation causing AT deficiency may provide useful information for relatives of index patients.

As mutation diagnostics evolves, new, more thorough methods are emerging. As recognised in other areas this can eventually lead to alternating results, which is problematic as DNA investigations in general are considered flawless and irrevocable. We here report on discordant diagnoses from two different investigation approaches, namely detection of AT sequence variation with denaturing high-performance liquid chromatography (DHPLC) followed by sequencing of pre-selected regions in contrast to direct sequencing of all *SERPINC1* exons and exon–intron boundaries without pre-selection by DHPLC.

* Corresponding author at: Dept. of Clinical Biochemistry and Pharmacology, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark. Fax: +45 6541 1911.
E-mail address: mads.nybo@rsyd.dk (M. Nybo).

http://dx.doi.org/10.1016/j.clinbiochem.2014.06.013
0009-9120/

Table 1
Biochemical and genetic findings in 16 patients initially found negative for mutations in the AT gene (*SERPINC1*) by DHPLC and sequencing. Numbers in brackets are reference intervals.

	Antithrombin activity[c]			Type of AT deficiency	Detection of mutations by DHPLC[a]	Detection of mutations by sequencing[b]	Disease-related mutation	Amino-acid change	Referred due to
	FIIa:L[d] (0.80–1.20)	FIIa:S[e] (0.80–1.20)	FXa[f] (0.80–1.20)						
Female 60 years old	0.99 (0.99/0.98)	0.71 (0.72/0.70)	0.85 (0.82/0.88)	IIb	No	Yes	c.218C>T	p.P73L	DVT × 3
Female 42 years old	1.05 (1.02/1.07)	0.70 (0.70/0.69)	0.88 (0.79/0.97)	IIb	No	Yes	c.218C>T	p.P73L	Arterial thrombosis
Female 34 years old	1.10 (1.13/1.07)	0.68 (0.62/0.74)	0.76 (0.77/0.75)	IIb	No	Yes	c.218C>T	p.P73L	DVT × 2
Male 33 years old	0.92 (0.90/0.94)	0.68 (0.72/0.64)	0.74 (0.74/0.73)	IIb	No	Yes	c.218C>T	p.P73L	Family history
Female 52 years old	0.91 (0.90/0.92)	0.69 (0.61/0.77)	0.72 (0.75/0.69)	IIb	No	Yes	c.218C>T	p.P73L	Family history
Male 50 years old	0.77 (0.74/0.80)	0.75 (0.71/0.79)	0.90 (0.82/0.98)	IIa	No	Yes	c.1246G>T	p.A416S	Venous thrombosis
Female 41 years old	0.97 (0.99/0.94)	0.72 (0.78/0.66)	1.01 (0.95/1.07)		No	No			Family history
Female 38 years old	0.80 (0.74/0.86)	0.70 (0.74/0.66)	0.98 (0.92/1.04)	IIa	No	Yes	c.1246G>T	p.A416S	DVT × 3
Female 62 years old	0.77 (0.72/0.82)	0.64 (0.53/0.74)	0.61 (0.58/0.64)		No	No			Family history
Male 43 years old	0.79 (0.79/0.78)	0.79 (0.82/0.76)	0.75 (0.77/0.72)		No	No			DVT
Male 40 years old	0.66 (0.56/0.75)	0.75 (0.78/0.72)	0.72 (0.72/0.72)	IIb	No	Yes	c.218C>T	p.P73L	Family history
Male 36 years old	0.71 (0.65/0.77)	0.69 (0.70/0.67)	0.76 (0.69/0.83)		No	No			DVT
Female 52 years old	0.67 (0.68/0.65)	0.67 (0.57/0.76)	0.63 (0.66/0.60)		No	No			Arterial thrombosis
Male 61 years old	0.62 (0.61/0.62)	0.66 (0.61/0.71)	0.77 (0.77/0.76)		No	No			Venous thrombosis
Female 51 years old	0.60 (0.56/0.63)	0.72 (0.73/0.70)	1.00 (1.00/1.01)	IIb	No	Yes	c.218C>T	p.P73L	Family history
Female 29 years old	0.63 (0.60/0.65)	0.69 (0.68/0.69)	1.03 (1.01/1.05)	IIb	No	Yes	c.218C>T	p.P73L	Arterial thrombosis

[a] DHPLC; DNA sequence variation in *SERPINC1* detected by DHPLC followed by sequence analysis of amplicons which appear heterozygous.
[b] Sequencing; DNA sequence variation detected by sequence analysis of all *SERPINC1* exons without pre-analysis by DHPLC.
[c] Mean of two measurements (shown in brackets).
[d] FIIa:L; AT activity measured using a FIIa-inhibition method with long incubation time, see Materials and methods.
[e] FIIa:S: AT activity measured using a FIIa-inhibition method with short incubation time, see Materials and methods.
[f] FXa; AT activity measured using a Xa-inhibition method, see Materials and methods.

Materials and methods

Patients

Sixteen patients with a personal or family history of venous or arterial thrombosis and with decreased antithrombin activity were enrolled in this study (Table 1). All patients were initially (i.e. using the DHPLC pre-selection approach) found negative for mutations in the AT gene (*SERPINC1*).

Measurement of antithrombin activity

AT activity was measured using three different methods: Two based on factor IIa (FIIa)-inhibition and one based on factor Xa (FXa)-inhibition. The FIIa-inhibitor methods will be referred to as FIIa:Long (FIIa:L) and FIIa:Short (FIIa:S). In the FIIa:L method, AT activity was measured on a STAR coagulometer (Diagnostica Stago, Amsieres-Sur-Seine, France) using the STA Antithrombin III reagents as described by the manufacturer. Pre-incubation time was 60 s. In the FIIa:S method, AT activity was measured as described earlier [6]. Pre-incubation time was 10 s. For the FXa-inhibition method AT activity was also measured on a STAR coagulometer using the Coamatic antithrombin reagents (Chromogenix, Mölndal, Sweden) as described by the manufacturer.

Antithrombin gene analyses

Blood samples were collected into EDTA tubes and genomic DNA (gDNA) was extracted from whole blood using a Maxwell 16 Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA). To identify sequence variation in the AT gene all seven *SERPINC1* exons were amplified (primer sequences are available upon request) from gDNA and amplicons were subjected to DHPLC on a WAVE® System (Transgenomic, Inc., Omaha, NE, USA). Amplicons, which by DHPLC appeared heterozygous at one or more of the applied temperatures, were subsequently sequenced bi-directionally on an ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). In addition to this approach all samples were analysed by direct sequencing of all amplified *SERPINC1* exons without any initial DHPLC pre-selection step. As described for the DHPLC approach amplified exons were bi-directionally sequenced on the ABI 3730xl DNA Analyser.

Results

All patients had biochemistry measurements indicating AT deficiency (Table 1). Interestingly, all had decreased AT activity when measured with a short incubation period (IIa:S), while only seven