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Denaturing High-performance Liquid Chromatography mutation analysis in patients with reduced Protein S levels

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Abstract

Background: Patients with congenital Protein S deficiency have increased risk of venous thromboembolism. However, Protein S levels show large intra-individual variation and the biochemical assays have low accuracy and a high interlaboratory variability. Genetic analysis might aid in a more precise diagnosis and risk estimation. The aim was to design a high-throughput genetic analysis based on denaturing high-performance liquid chromatography to identify sequence variations in the gene coding for Protein S.
Patients: In total, 55 patients referred to the Section of Thrombosis and Haemostasis, Odense University Hospital, in the period 1998–2004 were included in the study.
Results: Mutations were found in ten of the 55 patients: Six different variants were identified, of which four were not previously reported: One were a nonsense mutation substituting a glutamine with a stopcodon (c.790C>T) and the rest were missense mutations (c.932T>G; c.1367A>G; c.1378T>C). Furthermore, four patients carried the same mutation (c.1045G>A), while two carried the Heerlen mutation (c.1378T>C).
Conclusions: The reported method will be useful for rapidly detecting sequence variations in the gene coding for Protein S, giving a precise diagnosis and subsequently a better risk estimation.

Keywords: DHPLC; Mutation detection; Protein S

1. Introduction

Protein S, a vitamin K-dependent plasma glycoprotein, is an important inhibitor of thrombosis by at least two different mechanisms. Protein S acts as a nonenzymatic cofactor for activated Protein C (APC). This complex provides an important control of blood coagulation by the degradation of membrane bound thrombin activated coagulation factor VIIIa (FVIIIa) and factor Va (FVa) [1]. Furthermore, new data shows that Protein S inhibits tissue factor activity by enhancing the interaction between tissue factor pathway inhibitor and coagulation factor FXa, thereby accelerating the feedback inhibition of the extrinsic coagulation pathway [2]. Two Protein S genes, *PROS1* and *PROSP,* exist in the human genome – both located on chromosome 3. The active gene, *PROS1,* exhibits 97% similarity with the coding region of the pseudogene *PROSP,* with the exception of exon 1 which is lacking in *PROSP.* The occurrence of *PROSP* makes genetic analysis of the *PROS1* gene difficult.

The biochemical assays measuring the concentration and the activity of Protein S have been used to diagnose patients with Protein S deficiency. However, the use of Protein S measurements are complicated by the fact that in human plasma, 30% to 40% of Protein S circulates as free protein, the remaining bound

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to complement component 4-binding protein (C4BP) [3]. As only the free Protein S has the ability to function as a cofactor for APC, this fraction of the total Protein S has the greatest clinical interest [4]. Three types of Protein S deficiencies have been defined using both immunological assays measuring the total and free Protein S antigen, and activity measurements with respect to APC cofactor function [5,6]. Type I deficiency is associated with a reduction in both total and free antigen levels (quantitative deficiency). Type II deficiency is associated with a reduced activity only (qualitative deficiency), and type III has total Protein S antigen levels within the normal range, but reduced free antigen levels [5]. Type I and type III deficiencies are probably phenotypic variants of the same disease [7].

Biochemical Protein S deficiency is very difficult to diagnose accurately as plasma levels are influenced by age, sex, liver disease, oral contraceptive use, pregnancy, and coumarin therapy [8–10]. Furthermore, there are large intraindividual variations as well as low accuracy in all biochemical assays used for quantification and a high interlaboratory variability [11].

Although it is generally accepted that Protein S deficiency results in an increased risk of venous thromboembolic disease [12,13], the estimates of the increased thromboembolic risk are very variable and have not been confirmed in all studies [12,14,15]. In contrast, there have been reports of hereditary Protein S deficiency with a history of recurrent acute myocardial infarction [16] and arterial thromboembolism among children [17,18].

The variable penetrance of Protein S deficiency may be explained by the presence of coexistent risk factors such as the factor V Leiden and the prothrombin 20210 mutations [11]. Factor V Leiden is the most common inherited risk factor for venous thrombosis and is a point mutation in the factor V gene, making the activated factor V molecule more resistant to the anticoagulant effect of activated protein C (APC resistance). Factor V Leiden is found in approximately 6.6% of the Danish population [19] and patients who are heterozygous or homozygous for this mutation have an approximately 3 fold and 18 fold, respectively, increased risk for thrombosis. The prothrombin 20210 mutation is the second most common inherited risk factor for venous thrombosis and the overall prevalence of heterozygotes are 2% in Caucasians. This mutation results in a slightly higher level of circulating prothrombin in heterozygous individuals.

Hereditary Protein S deficiency is an autosomal dominant trait and the prevalence of hereditary Protein S deficiency is estimated to be between 0.03% and 0.13% [20]. However, the relatively high frequency of heritable Protein S deficiency in patient populations with venous thromboembolism in combination with the highly variable biochemical assays, the need for diagnostic tools during pregnancy and in patients receiving oral anticoagulant treatment (OAC) combined with the need for genetic counselling of family members, underline the desirability of a genetic based analysis. The method described in this study is a high throughput procedure based on denaturing high-

Table 1
Primers, PCR and DHPLC conditions

Exon	Primer	Sequence	PCR °C	mM $MgCl_2$	DHPLC temperatures
Exon 1	Ps.ex1.sn	CGAAAAGCTTCCTGG	50	2.0	60.2; 61.0; 65.3; 68.0; 69.5
	Ps.ex1.asn	TGCAGCTCTAGAGAA			
Exon 2 [a]	PSex2sn	AAATGTCATACAATTCATAGGCAG	58	1.5	51.4; 54.5; 56.5; 57.6
	PSex2ans	CAGAAGGAAGTACAGGCTGG			
Exon 3 [a]	PSex3sn	AATTATAATGTGAAAATGATGGTTATATG	58	2.0	51.1; 52.1; 53.1; 54.1
	PSex3ans	TAGATAGGTGGAGAGTTAGACAGGA			
Exon 4	PSex4sn	TTCAGATCAAGTATGTGTGTCTACTCT	58	1.5	54.7; 56.7; 58.5
	PSex4ans	CCATGGGTGTACTTTACCTACAGA			
Exon 5+6	PSex5+6sn	ATTTTATTTTTCCATGACATGAGAT	58	1.5	52.9; 54.1; 55.1; 57.6
	PSex5+6ans	TCTCTAACTGGGATTATTCTCACAT			
Exon 7	PSex7sn	ACACAAATCAAGGGTTCTTTGG	58	1.5	54.3; 57.6; 58.5
	PSex7ans	CAAAGCCAATGCTTTTAAATATCAG			
Exon 8	PSex8sn	GGGGATATTAAAGTTTGTGTGC	58	1.5	55.2; 56.2; 58.0
	PSex8ans	GAACGTCTGTATTTTCCTGACTTAG			
Exon 9	PSex9sn	CACACAAACATTAAGCAATAACCTG	58	1.5	53.0; 54.8; 56.6
	PSex9ans	CTAGAATGACTCAAAAAGGTTTTAGG			
Exon 10	PSex10sn	CATTGAGCTTTCTGTATTTCTTACTC	58	1.5	54.3; 55.3; 56.1; 59.1
	PSex10ans	AATCCATTTTGGTTTGGTATCA			
Exon 11	PSex11sn	TAGATCCCAGTATTTCAAATGACAT	58	1.5	53.1; 54.1; 55.1; 57.0
	PSex11ans	TCAGAAACACACATATTCAAATCTATTAC			
Exon 12	PSex12sn	TTAAAATGCACTCCTTGACTTG	58	1.5	52.8; 53.9; 56.3
	PSex12ans	GAGTGGGCACACAGTAGATACTC			
Exon 13	PSex13sn	GATCATTGAGAAAGGGAATGG	58	1.5	52.8; 54.5; 57.5; 59.2
	PSex13ans	AATGAAAAGAAAAACAAGGCTTATA			
Exon 14	PSex14sn	GACTTTCAAAAACTCAAAAGTCACT	58	2.0	51.3; 52.0; 55.2; 56.7; 58.0
	PSex14ans	TGTCGGTACTAGCCCCTAGAAA			
Exon 15	PSex15sn	TAGGATTAGAATTTGGTTGGAAAC	58	1.5	52.7; 54.3; 54.8; 55.7; 56.7
	PSex15ans	TCACTATTCTTAGATAGCAAGAGAAGTAAG			

The underlined part of the primers is specific for *PROS1* and deviates from the pseudogene. The annealing temperature, $MgCl_2$ concentration and DHPLC temperatures are shown.

[a] Primers chosen from Simmonds et al. [23].