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Regular Article

Hereditary protein C deficiency caused by the Ala267Thr mutation in the protein C gene is associated with symptomatic and asymptomatic venous thrombosis

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ABSTRACT

Introduction: Protein C (PC) is a key anticoagulant that regulates hemostasis, and inherited deficiency of PC is an established risk factor for venous thrombosis (VT). The factor V Leiden mutation causing activated PC (APC) resistance is an additional risk factor for VT. Reduced PC levels in the circulation and/or APC resistance do not necessarily lead to thrombotic disease. In the present study we describe and characterize an ethnic Lebanese family in which individuals with reduced PC levels and APC resistance have various clinical symptoms.

Methods: PC activity and antigen levels and APC resistance in the family members were quantified with commercial kits. Sequencing of PC DNA and mRNA was performed with BigDye Terminator Cycle Sequencing kit on the ABI 3730 Genetic Analyzer.

Results: PC antigen and anticoagulant activity in the plasma of the proband and family members ranged from 9% to 69% and 3% to 63%, respectively, compared to levels measured in pooled normal plasma. Sequencing analysis of the PC gene of family members revealed that they were either homozygous or heterozygous for the Ala267Thr mutation. In addition, three of them exhibited APC resistance. None of the family members, except the proband, have had a history of VT despite that two of them have two genetic risk factors for thrombosis.

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Introduction

Protein C (PC) is a vitamin K-dependent plasma glycoprotein and a precursor of the anticoagulant serine proteinase that inhibits blood coagulation by proteolytic inactivation of factors Va and VIIIa [1]. Individuals with moderate PC deficiency are at increased risk for severe thrombotic disorders, mostly venous thrombosis (VT) [2–4]. Very low plasma PC levels (<1% of normal levels) are usually not compatible with life, as individuals with such low levels suffer from life-threatening thrombotic complications immediately after birth [5–7]. However, several cases of PC deficiency in patients with very low, but measurable PC levels, and thrombotic manifestations, which started later during childhood or early adult life have been described [8,9].

The human PC (PROC) gene is located on chromosome 2q13-q14 and consists of nine exons with 8 introns spanning 10.8 kb of genomic DNA [10,11]. Around 200 different mutations in the PROC gene have so far been reported in the PROC database [12]. Several of the mutations have been characterized and some of them have been found to be associated with reduced levels of PC in plasma, i.e., PC deficiency [13]. In addition to mutations in the coding region, sequence variations in the promoter region of the PROC gene have been reported to be associated with altered plasma PC levels and risk of thrombosis [14,15].

Based on laboratory evaluation of PC activity and antigen levels in plasma, PC deficiency can be characterized as either heterozygous or homozygous. Patients with heterozygous PC deficiency have PC activity and antigen levels from 35% to 65% of normal levels [13,16]. Individuals who inherit double abnormal allele for PROC gene mutation, one from each parent, are characterized as homozygous for the mutation. Although it may be appropriate to assume that patients homozygous for the mutation have lower antigen and activity levels than those heterozygous for the mutation, it is not always the case as subjects with a homozygous PROC gene mutation and heterozygous PC deficiency have been described [17].

PC deficiency can also be found in association with other genetic defects predisposing for thrombosis [18] including activated PC (APC) resistance due to the factor V G1691A gene mutation, also called the factor V Leiden mutation, and the prothrombin G20210A gene mutation [19,20]. It has been shown that individuals with both PC deficiency and APC resistance have a higher risk for developing thrombosis at younger age as compared with carriers of PC deficiency alone [21,22].

The present work describes the clinical features and the results of molecular genetic analysis of a Lebanese family with PC deficiency. The proband was a woman 25 years of age with recurrent deep vein thrombosis and reduced PC activity and antigen levels. Six of her

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immediate family members were PC deficient. Sequencing of the PROC gene revealed that the proband and some members of her family were either homozygous or heterozygous for the Ala267Thr mutation. Three of them exhibited also APC resistance due to the factor V Leiden mutation. Except for the proband, none of these individuals have so far suffered from thrombosis.

Materials and methods

Study subjects

The subjects in the study, all of Lebanese origin, are shown in Fig. 1. The mother and the father were first cousins. After a period with gastroenteritis and dehydration, the proband developed massive, proximal deep vein thrombosis in the left leg at the age of 16. Subsequent to initial treatment with intravenous streptokinase and unfractionated heparin, she developed skin necrosis in the left foot after approximately 4 weeks on warfarin. The PC activity and antigen levels, while still on warfarin, were <1%. Warfarin was discontinued and long-term treatment with low molecular weight heparin was initiated. After 2 years on low molecular weight heparin she was reinstalled on warfarin due to low compliance on low molecular weight heparin. Although she was covered by prophylactic doses of low molecular weight heparin, she developed fulminant disseminated intravascular coagulation that was successfully treated using therapeutic doses of intravenous heparin and PC concentrate (Ceprotin, Baxter AG, Vienna, Austria). She has later been on low molecular weight heparin prophylaxis using half therapeutic dose. During her first pregnancy she developed renal vein thrombosis despite prophylaxis with low molecular heparin. Her siblings, parents and daughter suffering from PC deficiency, have so far been asymptomatic without any evidence of VT. In the present study, written informed consent was obtained from all subjects.

Blood sampling and assays

Whole blood was collected in 5 ml Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing either 0.5 ml buffered citrate or K_2-EDTA. Citrated plasma was separated immediately after collection by centrifugation 2000 ×g for 15 min at room temperature and stored in aliquots at -70 °C. Genomic DNA was extracted from EDTA whole blood by automatic isolation on MagNA Pure instrument using MagNA Pure LC DNA Isolation Large Volume kit (both Roche Diagnostics GmbH, Mannheim, Germany). Samples for DNA extractions were available from I-1, I-2, II-2, II-4, II-5, II-6 and III-1. As indicated in Fig. 1, no samples from the father of the proband's offspring (III-1) were available, but he was a relative of the proband. Neither were samples available from the proband's second child.

PC activity, PC antigen and APC resistance were assayed in citrated plasma using commercial reagent kits. PC activity was determined using the Coamatic® Protein C assay (Chromogenix, Mölndal, Sweden) run on ACL Futura (Instrumentation Laboratory, Milan, Italy). PC antigen was assayed using the Elisara Protein C kit (Aniara Corporation, Mason, OH). APC resistance in plasma was assayed using the Pefakit® APC-R Factor V Leiden kit (Pentapharm Ltd., Bassel, Switzerland). The factor V Leiden and the prothrombin gene G20210A mutations were detected by commercial kits run on LightCycler 2.0 (all from Roche Diagnostics).

Amplification and sequencing of the PROC gene

All nine exons of the PROC gene were PCR amplified using oligonucleotide primers as described previously [23] with the exception that the primers used to amplify exon 6 were as follows: forward 5′-CCTCCCCTGCCCGCAGA-3′; reverse 5′-CGTGATTCCTGGGC-GATGTATT-3′. The amplified fragments covered the entire coding sequence and the intron-exon splice junctions. The primers were purchased from TIB MOLBIOL (TIB MOLBIOL GmbH, Berlin, Germany). The PCR amplifications were carried out on GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems, Foster City, CA) using the Taq PCR Master Mix kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A fragment in the promoter region of the PROC gene covering nucleotide positions –1733 to –1359 was amplified using the following primers: forward 5′-CTCCCTCAGCCAGCCACTAT-3′ and reverse 5′-GATTCAGCCCTCATCACCAC-3′ (Eurogentec, Seraing, Belgium). The sequencing reactions were carried out in a total volume of 10 µl using ABI PRISM Big Dye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's protocol. The sequencing primers were the same as for the PCR amplifications. The products were purified using the Montáge® SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA) according to the manufacturer's protocol and run on ABI Prism 3730 (Applied Biosystems). The sequences were analysed with the SeqScape Software, version 2.5 (Applied Biosystems). The numbering of the all PC nucleotides was done according to Foster et al [10].

Sequencing of mRNA

Total RNA was extracted from blood using the PAXgene™ Blood RNA System Kit (Qiagen) employing the manufacturer's guidelines. The

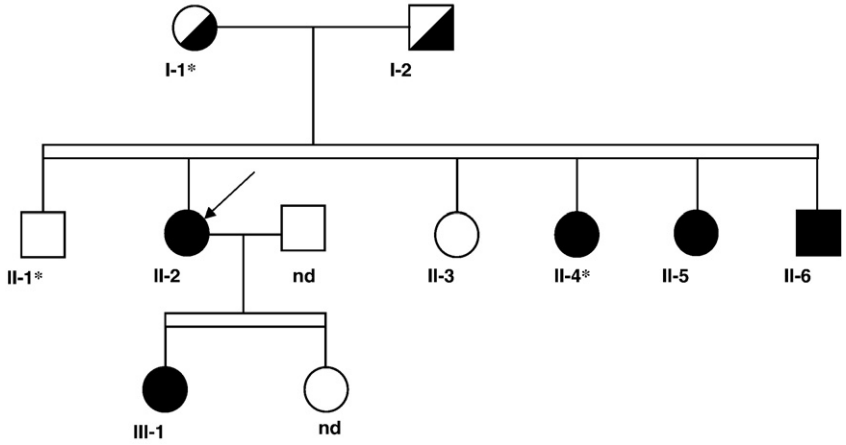

Fig. 1. The pedigree of the family with protein C (PC) deficiency. Half-solid and solid symbols represent individuals heterozygous and homozygous for the Ala267Thr mutation, respectively. The sibships are indicated by a double line. The proband is indicated with an arrow. *= individuals with APC resistance; nd= not determined (samples not available).

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