Gene xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Gene

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

Research paper

Compound heterozygous protein C deficiency in a family with venous thrombosis: Identification and *in vitro* study of p.Asp297His and p.Val420Leu mutations

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ARTICLE INFO

Article history:
Received 9 November 2014
Received in revised form 5 February 2015
Accepted 2 March 2015
Available online xxxx

Keywords:
Protein C deficiency
Mutation
Venous thromboembolism (VTE)

ABSTRACT

Hereditary protein C deficiency (PCD) is an autosomal inherited disorder associated with high risk for venous thromboembolism (VTE). This study aimed to explore the functional consequences of two missense mutations, p.Asp297His and p.Val420Ile, responsible for type I/II PCD and recurrent deep vein thrombosis (DVT) in a Chinese family. The plasma protein C activities (PC:A) of the proband and his sister were reduced to 4% and 5% of normal activity. However, protein C antigen (PC:Ag) concentrations were not equally decreased, with levels of 90.5% and 88.7%, respectively. Two missense mutations p.Asp297His and p.Val420Leu were identified in the protein C gene (*PROC*). The PC:A and PC:Ag levels in heterozygous state for p.Asp297His were 66% and 64.8%, whereas in heterozygous state for p.Val420Leu, these levels were 67% and 145%, respectively. Wild type (WT) and two mutant *PROC* cDNA expression plasmids were constructed and transfected into HEK 293T cells. Western blot analysis revealed that both p.Asp297His and p.Val420Leu showed a normal intracellular protein level. The extracellular protein level and specific activity of p.Asp297His were equally reduced to 37.7 ± 4.3% and 22.1 ± 2.5%, respectively. Mutant p.Val420Leu showed a relatively higher PC:Ag level and undetectable PC:A. Immunofluorescence staining revealed that WT and p.Val420Leu proteins were largely co-localized with both the protein disulfide isomerase (PDI) and *cis*–Golgi Marker (GM130), while the PC p.Asp297His mutant protein was mainly co-localized with PDI and much less co-localized with GM130. The thrombosis symptom in this family was associated with the two missense mutations in the *PROC* gene.

1. Introduction

Protein C (PC) is a vitamin-K dependent serine protease zymogen synthesized mainly in the liver. Activated PC (APC) is generated upon the zymogen proteolysis between Arg169 and Leu170 on the surface of endothelial cells. Thrombin, thrombomodulin and endothelial protein C receptor (EPCR) play a role during the process (Wildhagen et al., 2011). APC exerts its anticoagulant function through inactivation of the blood coagulation factors FVa and FVIIIa in the presence of protein S (PS), Ca^{2+} and phospholipids (Griffin et al., 2007).

PC is encoded by the protein C gene (*PROC*) on chromosome 2q13–q14, which is composed of 9 exons and spans about 11.2 kb (Cooper et al., 2012). Hereditary protein C deficiency (PCD) is an autosomal inherited disorder with an increased risk of venous thrombosis (Vossen et al., 2004). Patients with undetectable protein C activity (PC:A) often present life-threatening thrombotic complications such as purpura fulminans and DIC during the neonatal period. Heterozygous PCD is often characterized by an increased risk of venous thrombosis in early adulthood (Ohga et al., 2013). Based on the functional and immunological protein C assays, PCD can be divided into two types. Type I deficiency is featured by a parallel reduction in concentration and function (CRM−), whereas type II is characterized by normal or increased concentration and reduced function (CRM+). Type II accounts for about 15% of symptomatic PCD (Faioni et al., 2000).

After synthesizing, PC is subjected to several posttranslational modifications in the endoplasmic reticulum (ER) and the Golgi apparatus, and only correctly folded proteins are transported from the ER to Golgi and subsequently to the cell surface (Trombetta and Parodi, 2003). Previous studies have shown that mutations in *PROC* leading to type I PC deficiency are due to retention of the misfolding mutant proteins in the ER. Some of the studies also detect increased degradation by proteasomes; the latter is called ER associated degradation (ERAD)

Abbreviations: PCD, hereditary protein C deficiency; VTE, venous thromboembolism; DVT, deep vein thrombosis; PC, protein C; ERAD, ER associated degradation; *PROC*, protein C gene; APC, activated PC; LMWH, low molecular weight heparin; UFH, unfractionated heparin

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http://dx.doi.org/10.1016/j.gene.2015.03.002
0378-1119/

(Nishio et al., 2008; Tjeldhorn et al., 2010). The present work describes a family with compound heterozygous mutations in the *PROC* gene.

2. Materials and methods

2.1. Clinical material

Subjects under study were the proband, his parents and two sisters. The proband suffered his first spontaneous attack of thrombosis at the age of 15 years affecting the right deep femoral vein. In the acute phase, the DVT was treated with low molecular weight heparin (LMWH) and followed by warfarin prescription, targeting an international normalized ratio of 2.0–3.0. Warfarin-induced skin necrosis and bleeding were not seen in this proband, but during the warfarin treatment the proband had several image-verified attacks of thrombosis affecting the left profound leg veins, the common iliac vein, the right internal jugular vein as well as a recurrence of thrombosis of his left profound femoral vein. The sister (III-2) had her first deep vein thrombosis in the right leg during pregnancy at the age of 20 years, and managed with LMWH and warfarin. She had no additional thrombosis attacks after the delivery. The blocked blood vessels of the proband and his sister (III-2) were not completely recanalized till now. For financial reasons, they refused to use new oral anticoagulants such as rivaroxaban and dabigatran. No history of thromboembolism was presented in other family members.

2.2. Sample collection and DNA extraction

Peripheral blood samples were collected from the family members after obtaining written informed consent. The platelet-poor plasma (PPP) was stored at −80 °C until assayed, and DNA was extracted from peripheral blood leukocytes according to the manufacturer's instructions (Bioteke, Beijing, China).

2.3. Ex vivo plasma measurements

PC, PS, and antithrombin (AT) activities were assayed on a STA-R automated coagulation analyzer (Diagnostica Stago, France) using commercial reagents from Stago according to the manufacturers' recommendations. PC and AT activities were measured using a chromogenic substrate method. The activity of PS was evaluated using a clotting method. PC antigen (PC:Ag) was further tested by ELISA using the ZYMUTEST Protein C Kit (Hyphen Biomed, France). The normal ranges of these tests in our lab were established in 78 healthy subjects.

2.4. Molecular analysis

After PCR amplification of the promoter region, nine exons, and the splicing regions of *PROC*, the PCR products were purified and directly sequenced (Applied Biosystems, USA). The results were compared with the reference sequences NM_000312.3 and NP_000303.1 in GenBank. The PCR primers and reaction conditions were available upon request. Novel variants were then screened in 50 healthy individuals with normal PC:A using direct sequencing.

2.5. In silico analysis of novel amino acid changes

The possible impact of novel coding sequence changes on the structure and function of PC was assessed using three bioinformatics tools: Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and MutationTaster (http://www.mutationtaster.org/). The gene ID "5624", the Protein ID "P04070" and ENSP ID "ENSP00000234071" were used.

The conservation of the affected amino acids were further checked by multiple sequence alignment (HomoloGene, http://www.ncbi.nlm.nih.gov/sites/entrez) with sequences from *Pan troglodytes*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, and *Gallus gallus*.

2.6. Recombinant PC expression experiments

Functional effects of p.Asp297His and p.Val420Leu were analyzed by *in vitro* expression studies. To produce the *PROC* mutants (c.889G>C, c.1258G>T), mutagenesis was performed by high-fidelity PCR with the wild-type human PC open reading frame expression-ready clone (GeneCopoeia, Rockville, MD, USA) as a template. Mutagenic primer sequences are available upon request. Each plasmid was checked by sequencing the whole protein C cDNA to confirm the presence of the mutation and to exclude PCR-induced errors. HEK-293T cells were grown in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FBS, 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, and maintained at 37 °C in 5% CO_2. Approximately 70–80% confluent cells in six-well plates were co-transfected with 2.0 μg of each *PROC* construct (monk vector, wild type or mutant type) and 0.5 μg of the enhanced green fluorescent protein (EGFP, GeneCopoeia, Rockville, Maryland, USA) control vector using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, California, USA). The EGFP was used for normalizing transfection efficiency. After 4 h transfection, the medium was changed to serum-free medium.

2.7. Expression levels of recombinant PC

The cells were harvested 48 h after transfection and proteins were prepared by lysing transfected cells and ultrafiltering mediums, respectively, separating by 7.5% SDS-PAGE, and transferring to PVDF membranes. Protein C was detected by Western blot analysis using a rabbit polyclonal antibody against human PC (Hyphen Biomed, France), followed by HRP-conjugated anti-rabbit antibody, and ECL detection reagents (Amersham Biosciences, Piscataway, New Jersey, USA). The GAPDH served as control. After 72 h transfection, the secreted PC:Ag concentrations of the wild-type and mutant PC present in culture media were measured by ELISA described above (see "*Ex vivo* plasma measurements"). The total protein concentration was measured by Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA). PC:Ag levels in cell lysates and culture medium were normalized against the total protein concentrations of the corresponding lysate samples.

2.8. Specific activity of recombinant PC

After 48 h transfection, the mediums were collected without protease inhibitor and concentrated using ultrafiltration tubes (Millipore, USA). PC activities were tested as described (see "*Ex vivo* plasma measurements"). The relative PC specific activity (expressed as a percentage of wild type, set as 100%) was calculated as the ratio between the PC:A and PC:Ag level.

2.9. Immunofluorescence

HEK-293T cells were grown on cover slips in the 24-well plate and transfected with 0.5 μg plasmid DNA. The cells were washed 3 times with PBS, fixed with 3.7% paraformaldehyde in PBS for half an hour at 4 °C, and permeated with 0.1% Triton-X (Sigma-Aldrich) in PBS for 20 min. Non-specific binding sites were blocked with 4% hydrogen peroxide for 20 min and 1% BSA for 20 min at room temperature. After removing the blocking solution, the cells were incubated with appropriate primary antibodies: polyclonal rabbit anti-human protein C antibody (Hyphen Biomed, France), mouse monoclonal anti-PDI (Abcam, UK) or mouse monoclonal anti-GM130 (Abcam, UK) diluted in PBS with 5% BSA. The cells were washed 3 times with PBS to remove unbound antibody and incubated in the dark for 1 h at 37 °C with Cy3 and FITC-conjugated secondary antibodies. The coverslips were removed from the well and mounted onto microscope slides after

Please cite this article as: Liu, H., et al., Compound heterozygous protein C deficiency in a family with venous thrombosis: Identification and *in vitro* study of p.Asp297His and p.Val420Leu mutations..., Gene (2015), http://dx.doi.org/10.1016/j.gene.2015.03.002