Different impact of two mutations of a novel compound heterozygous protein C deficiency with late onset thrombosis


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ABSTRACT. We investigated the alteration of coagulation state in a protein C (PC) deficiency pedigree and the impact of the PC gene mutations. The pedigree of a proband with cerebral hemorrhagic infarction had sixteen members with four generations. The plasma levels of PC activity (PC:A), protein S activity (PS:A), factor V:C and factor VIII:C, and routine coagulation tests were measured. Nine exons of the PC gene (PROC) were sequenced. Plasma PC:A and PC antigen (PC:Ag) of the proband were 26 and 18%, respectively, which was significantly lower than normal ranges. Two heterozygous missense mutations of PC in the proband were identified, T>G at site 6128 (exon 7) and G>C at site 8478 (exon 9) resulting in F139V and D255H, respectively. The family members with F139V (N = 4) or D255H (N = 4) had lower levels of PC:A and PC:Ag than members with wild-type
PROC (N = 6). D255H mutation caused a more significant decrease in the levels of PC:A, PC:Ag and factor V:C as compared to F139V mutation (P < 0.05). Two independent mutations, F139V and D255H, of PROC reduce PC function. Compound heterozygous condition of the two mutations can cause synergistic PC deficiency, but resulting in later onset of cerebral thrombosis.

**Key words:** Compound heterozygous gene mutation; Thrombosis; Protein C deficiency; Pedigree

**INTRODUCTION**

Protein C (PC) is a vitamin K-dependent serine protease, which is synthesized and secreted by liver cells as a zymogen. The role played by activated PC in downregulating the blood coagulation cascade is through the proteolytic effect on factors Va and VIIIa, in conjunction with its cofactor protein S. Furthermore, PC proteolytically inactivates the tissue plasminogen activator inhibitor-1, thereby increasing the natural fibrinolytic activity of plasma (Foley et al., 2012). Hereditary PC deficiency causes the condition of the hypercoagulable state, which was first described in 1981 by Griffin et al. As an autosomally inherited disorder, hereditary PC deficiency can be caused by a wide variety of mutations in the PC gene (PROC), including missense mutations, nonsense mutations, frame-shift mutations, and splice-site abnormalities. These genetic changes commonly lead to significant intracellular degradation and inefficient secretion of mutated PC variants compared to wild-type PC (Sugahara et al., 1994; Naito et al., 2003; Tjeldhorn et al., 2010) or secretion of non-functional PC (Greengard et al., 1994).

PROC contains nine exons, which produce the precursor of PC. It contains a 42-amino acid preproleader sequence, a 155-amino acid light chain, a connecting dipeptide of Lys-Arg, and a heavy chain of 262 amino acids (Foster et al., 1985). The first and part of the second exon are non-protein coding sequences. Mutations in this region can cause reduced transcription of PC. The missense mutations in the functional regions of light chain or heavy chain can result in abnormal levels of protein C antigen (PC:Ag) and protein C activity (PC:A) (Greengard et al., 1994).

Clinical presentation of PC deficiency is variable (Aiach and Gandrille, 1996), associated with an increased risk of venous thromboembolic complications in young adulthood without apparent cause (Khor and Van Cott, 2010), such as deep vein thrombosis and pulmonary embolism. An estimated prevalence of the heritable PC deficiency is reported ranging from 0.2 to 0.5% (Esmon, 1987; Miletich et al., 1987). Many individuals affected with PC deficiency from a heterozygous mutation remain asymptomatic for life (Miletich et al., 1987; Zhu et al., 2011). The prevalence of symptomatic hereditary PC deficiency in the population is estimated between 1:16,000 and 1:36,000 (Formstone et al., 1996). Homozygous and compound heterozygous PC deficiency (CHPD) is very rare, which can result in neonatal purpura fulminans, or life-threatening thrombotic disorder (Seligsohn et al., 1984; Ido et al., 1993). PC deficiency is mainly diagnosed by evaluation of plasma PC:Ag and PC:A levels. Phenotypically, protein C deficiency can be classified into two types. Type I deficiency is more common, characterized by a simultaneous reduction in PC:A and PC:Ag levels. In type II deficiency, PC:A is reduced to a greater extent, and is non-parallel to the quantity of PC:Ag (Berdeaux et al., 1993; Tridapalli et al., 2010).
In the present study, a family pedigree of hereditary PC deficiency was investigated. We characterized a compound heterogeneous PC deficiency in a Chinese family. Two mutations of PROC alleles derived separately from the respective parents were identified and resulted in type I PC deficiency. The impact of the two respective mutations on coagulation tests including PC:A and PC:Ag was analyzed.

MATERIAL AND METHODS

Subjects

There were 16 subjects from 4 generations of one family in the study. One proband with hereditary deficiency of PC was diagnosis by the coagulation test, and 9 exons of PROC sequenced. Detailed medical history data of families on previous episodes of venous thromboembolism (VTE), liver disease, renal disease, severe infection, and anticoagulant treatment were collected by using a standardized questionnaire, and reviewing medical records. Blood samples of family members were collected in vacuum tubes containing 0.109 M sodium citrate after clinical data had been collected. The study was approved by the institutional review board of the First Affiliated Hospital of Wenzhou Medical College.

Laboratory studies

Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), factor Va, and factor VIIIa were detected by a clotting assay. Fibrinogen (FIB) was detected by the Von Clauss method. D-dimer (D-D) was determined by immunoassay. PC:A, protein S activity (PS:A), and antithrombin activity (AT:A) in plasma were measured with chromogenic assays. These tests were conducted in the STAGO STA-R automated coagulation instrument (Diagnostica Stago, Asnieres, France) using a commercial kit (Diagnostica Stago). PC:Ag levels were measured by ELISA (Xitan Biotech Co., Shanghai, China).

Genomic DNA was extracted using the QIAamp DNA Blood Mini kit (QIAGEN, China). Nine exons of PROC were amplified by PCR and sequenced. Primers for sequencing PROC were synthesized (Invitrogen, Shanghai, China) and are listed in Table 1. PCR was performed in a total volume of 25 μL with 1X PCR buffer, 200 nM of each primer, and 12.5 U Taq DNA polymerase (Tiangeng Biotech Co., Beijing, China). This mixture was amplified for 40 cycles in an ABI 2720 thermal cycler (Applied Biosystems, CA, USA). The PCR protocol consisted of 40 cycles at 94°C for 30 s, annealing for 30 s (58°-64°C), and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were electrophoresed on agarose gels with ethidium bromide and scanned for target bands using a Nucleo-Vision imaging workstation.

Subtype of PC deficiency

Protein C deficiency types I and II were defined by reduced levels of protein C antigen and/or activity. PC:A divided by PC:Ag >0.7 suggests type I deficiency; <0.7 suggests type II deficiency (Reitsma, 1997; Hoshi et al., 2007).
Statistical analysis

Data were analyzed by SPSS version 11.5 and are reported as means ± standard deviation (SD), except for categorical data, which are reported as percentages (%). Statistical significance of differences was determined by one-way ANOVA. For all analyses, statistical significance was defined as P < 0.05.

RESULTS

Subjects

A 30-year-old female patient was sent to the emergency room presenting sudden onset of right-sided weakness, headache, and seizures. Cerebral computed tomography (CT) and magnetic resonance imaging (MRI) scans showed hemorrhage in the bilateral parietal lobe, and MRI and magnetic resonance venography (MRV) also showed mild gyral edema and superior sagittal sinus thrombosis. Laboratory testing for thrombophilic states, showed PC:A = 26%, PC:Ag = 18.60%, PT = 13.1 s, APTT = 27.7 s, FIB = 3.62 g/L, D-D = 1.24 mg/L, FVIII:C = 143%, FV:C = 120%. On the basis of symptoms and laboratory tests, the patient was diagnosed with hereditary PC deficiency and cerebral hemorrhagic infarction. After a 2-week treatment with heparin, followed by oral anticoagulant (warfarin potassium), the proband was discharged without previous symptoms, and no thrombosis in the superior sagittal sinus was found by MRV. No warfarin-induced skin necrosis was observed during a 12-month follow-up.

Except for the proband, only one member had symptoms of VTE. Two family members were diagnosed with hypertension and type II diabetes.

Mutation scan of PROC

Sixteen family members were scanned over 9 exons of PROC by sequencing. The missense mutations of PROC in the proband were identified as T>G at site 6128 (exon 7) and G>C at site 8478 (exon 9), resulting in F139V and D255H, respectively (Figure 1). The mutation of F139V was from the paternal side, and D255H was from the maternal side.
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proband was identified as a CHPD from non-consanguineous parents (Figure 2). In the other family members, six members were wild-type for PROC, four were identified with heterozygous *F139V*, and another four with heterozygous *D255H*.

![Pedigree of the family.](image)

**Figure 1.** Pedigree of the family.

![Mutations of PROC in proband with compound heterozygous protein C deficiency. The mutations of PROC were detected by sequencing. T6128G (*F139V*) in exon 7 and G8478C (*D255H*) in exon 9 were identified in a proband with compound heterozygous protein C deficiency (arrow).](image)

**Figure 2.** Mutations of PROC in proband with compound heterozygous protein C deficiency. The mutations of PROC were detected by sequencing. T6128G (*F139V*) in exon 7 and G8478C (*D255H*) in exon 9 were identified in a proband with compound heterozygous protein C deficiency (arrow).

**Coagulation test**

Both single mutations of *F139V* or *D255H* caused a decrease in PC:A and PC:Ag in the family members. *F139V* heterozygous PC deficiency family members (N = 4) had lower levels of
PC:A and PC:Ag than those with wild-type PROC (N = 6) (67.25 ± 5.38 vs 110.17 ± 19.28%; 68.59 ± 9.71 vs 100.13 ± 4.41%; P < 0.001). D255H heterozygous PC deficiency individuals (N = 4) also had lower levels of PC:A, PC:Ag, and factor V than individuals without mutation (N = 6) (46.25 ± 8.50 vs 110.17 ± 19.28%; 47.48 ± 8.11 vs 100.13 ± 4.41%; 87.00 ± 8.25 vs 112.67 ± 19.94%, P < 0.05). Moreover, D255H mutation carriers had significantly decreased levels of PC:A, PC:Ag, and factor V:C compared to F139V individuals (P < 0.05) (Figure 3). According to the decrease in levels of PC:A and PC:Ag, which paralleled each other, both mutations of PROC caused type I PC deficiency (Table 2). The coagulation test for levels of PS:A, PT, aPTT, TT, FIB, and D-D revealed no differences between family members with wild-type PROC and with the mutation.

![Figure 3](image)

**Figure 3.** Coagulation tests of family members, family members carrying the heterogeneous mutation F139V or D255H have lower levels of PC:A and PC:Ag. D255H family members showed more significant impact on PC:Ag, factors V:C and VIII:C, compared to individuals with F139V. *P < 0.001, vs wild group; #P = 0.058, vs F139V group; $P < 0.01, vs F139V group; ^P < 0.05, vs F139V group; %P < 0.05, vs wild group.

**Table 2.** Coagulation test and PROC mutations.

<table>
<thead>
<tr>
<th>Family members</th>
<th>Age (years)</th>
<th>Disease</th>
<th>PC:A (%)</th>
<th>PC:Ag (%)</th>
<th>PS:A (%)</th>
<th>AT:A (%)</th>
<th>F139V</th>
<th>D255H</th>
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<tbody>
<tr>
<td>I</td>
<td>88</td>
<td>N</td>
<td>66</td>
<td>62.25</td>
<td>72</td>
<td>96</td>
<td>+</td>
<td>-</td>
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<tr>
<td>II1</td>
<td>75</td>
<td>Hypertension</td>
<td>101</td>
<td>96.60</td>
<td>79</td>
<td>100</td>
<td>-</td>
<td>-</td>
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<tr>
<td>II2</td>
<td>72</td>
<td>VTE</td>
<td>43</td>
<td>49.55</td>
<td>74</td>
<td>92</td>
<td>+</td>
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<tr>
<td>II3</td>
<td>65</td>
<td>N</td>
<td>141</td>
<td>107.70</td>
<td>97</td>
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<td>99.20</td>
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<td>N</td>
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<td>91</td>
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<td>60</td>
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<td>II10</td>
<td>51</td>
<td>N</td>
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<td>81.15</td>
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<tr>
<td>II11</td>
<td>48</td>
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<td>30</td>
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<tr>
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<td>-</td>
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<td>5</td>
<td>N</td>
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<td>35.50</td>
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<td>80-120</td>
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VTE = venous thromboembolism. Superior sagittal sinus thrombosis: (+) = positive of the mutation; (-) = negative of the mutation.
DISCUSSION

Protein C deficiency can have an important influence on the physiological function of hemostasis, since activated PC plays a key role in the complex regulatory system of coagulation. The clinical importance of PC deficiency is associated with thromboembolic disease. In the present study, we found a female adult with cerebral thrombosis, identified as CHPD with mutations in T6128G (F139→V139) and G8478C (D255→H255). The proband indicated a significant deficiency of PC:A and low level of PC:Ag. The coagulation test and pedigree analysis, F139V or D255H mutation carriers in the family showed lower levels of PC:A and PC:Ag, compared to family members with wild-type PROC. Furthermore, the D255H mutation had a more significant influence on PC:A between the two mutations.

Hereditary PC deficiency should always be sought as an explanation for sudden onset thrombotic disorders in the newborn or young adult. Although autosomal recessive PC deficiency is rare, caused by homozygous or compound heterozygous mutations in PROC, it usually leads to life-threatening disease, such as purpura fulminans, a thrombosis associated with disseminated intravascular coagulation in neonates (Sugahara et al., 1992). In the present study, coagulation tests for PC:A and PC:Ag combination with genetic analysis for PROC mutations provided a substantial molecular confirmation of CHPD. However, the PC level of the neonate was only 15% compared to the adults, the first observed onset of thrombosis of the proband with CHPD was at 30 years old, and no symptoms of thromboembolic events at birth had been observed. This condition indicated that the compound heterozygous mutations caused mild PC deficiency, inconsistent with most previous reports of CHPD (Manabe and Matsuda, 1985; Deguchi et al., 1992; Miyata et al., 1996).

Thrombosis often occurs spontaneously in those with PC deficiency. CT and MRI facilitate the discovery and location of the thrombosis in a variety of organs, especially cerebral venous sinus thrombosis (CVST). The incidence of cerebral thrombosis and hemorrhage caused by PC deficiency is relatively low, as are the VTE complications of the lower extremities. Due to pregnancy, puerperium and oral contraceptive use, CVST commonly occurs in women between the ages of 20 to 35 years. Those multiple factors influence the form of CVST. In addition, PC deficiency is a key factor that increases the risk of recurrence of CVST.

According to the criteria of the subtypes of PC deficiency, both F139V and D255H caused type I PC deficiency. The main reason is that the mutations occurred in the hydrophobic core of PC, which causes the intracellular disintegration of mutated alleles. At present, the database of PROC mutations (www.hgmd.cf.ac.uk/ac/gene.php?gene=PROC) has registered 310 mutations or polymorphisms. F139V has been found in the PC deficiency pedigrees of Chinese and Japanese (Miyata et al., 1996; Zhou et al., 2007). D255H has also been reported in patients with PC deficiency and venous thrombosis (Tsay and Shen, 2004). The family members with the D255H heterogeneous mutation had lower levels of PC:A and PC:Ag compared to those members with F139V; suggesting that the D255H mutation impacts the quantity of PC more significantly. CHPD with F139V and D255H demonstrated a synergistic effect, decreasing PC function to 26%. Usually, homozygous or compound heterozygous mutations in PROC result in PC function reduced to below 5%. Although the PC deficiency can reduce the function of inactivation of factor VIIIa and Va, the change in VIII:C and V:C in the present study was not consistent between the family members with F139V or D255H. As part of the coagulation pitfall, VIII:C and V:C could be influenced by multiple factors.
In summary, compound heterozygous PC deficiency can also result in a late onset of thrombosis in the superior sagittal sinus. According to coagulation tests, the heterozygous F139V mutation slightly influenced PC:A and PC:Ag, which may potentially explain late onset. D255H mutation demonstrated more significant impact on hypercoagulation. The significance of PROC mutations in the coagulation test needs further study to identify the molecular pathogenesis.

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