The Clinical Presentation and Genotype of Protein C Deficiency with Double Mutations of the Protein C Gene

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Abbreviations list

PC	protein C
PF	purpura fulminans
PS	protein S
FV Leiden	factor V G1691A
FII variant	prothrombin G20210A
PROC	protein C gene
Rr	reference range
PCR	polymerase chain reaction
Pt(s)	Patient(s)

ICTH	intracranial thrombosis and hemorrhage
DVT	deep vein thrombosis

1

Abstract

Background. Severe protein C (PC) deficiency is a rare heritable thrombophilia leading to the thromboembolic events during the neonatal period. It remains unclear how complete PC gene (*PROC*) defective individuals develop or escape the neonatal stroke or purpura fulminans. ***Procedure***. We studied the onset of the disease and the genotype of 22 PC-deficient patients with double mutations in *PROC* based on our cohort (n=12) and the previous reports (n=10) in Japan. ***Results***. Twenty-two patients in 20 unrelated families had 4 homozygous and 18 compound heterozygous mutations. Sixteen newborns presented with purpura fulminans (n=11, 69%), intracranial thromboembolism and hemorrhaging (n=13, 81%), or both (n=8, 50%), with most showing a plasma PC activity of <10%. Six others first developed overt thromboembolism when they were over 15 years of age, showing a median PC activity of 31% (range: 19-52%). Fifteen of the 22 patients (68%) had the 5 major mutations (G423VfsX82, V339M, R211W, M406I, and F181V) or 2 others (E68K and K193del) that have been reported in Japan. Three of the 6 late-onset cases, but none of the 16 neonatal cases, had the K193del mutation, which has been reported to be the most common variant of Chinese thrombophilia. A novel mutation of A309V was determined in a late-onset family of two patients. ***Conclusions***. The genotype of double-*PROC* mutants might show less diversity than heterozygous mutants in terms of the timing of the onset of thrombophilia (newborn-onset or late-onset).

Introduction

The development of thromboembolism depends on the genetic background of patients and triggering factors. The presence of protein C (PC), protein S (PS) and antithrombin deficiencies is associated with a high risk of inherited thrombophilia. Patients who are heterozygous for either gene mutation develop venous thromboembolism earlier than the carriers of factor V G1691A (FV Leiden) or prothrombin G20210A (FII variant) [1]. In contrast to the high allelic frequency of FV Leiden or FII variant in Caucasians, neither of these mutations has been found in Asian patients. These natural anti-coagulant deficiencies are thus the leading genetic cause of Asian thrombophilia [2]. Recent studies [3,4] and a nationwide survey [5] in Japan have shown that the major inherited thrombophilia in pediatric patients is protein C (PC) deficiency, while protein S (PS) deficiency mainly affects adult patients due to the high prevalence of PS-Tokushima K196E [6,7].

Severe PC deficiency is an extremely rare thrombophilia due to complete PC defects, presenting as neonatal purpura fulminans (PF) in both Asian and Caucasian populations. Infants with double mutations of the PC gene (*PROC*) develop neonatal PF and/or stroke [8,9] and rarely escape the development of thromboses during infancy and childhood. Fetal distress and infections can predispose individuals who are heterozygous for *PROC* mutations to neonatal and pediatric thromboses [10-12]. However, little information is available on the onset of disease in PC-deficient individuals who harbor double mutations of *PROC*.

We herein report the first presentation and genotype of patients with biallelic *PROC* mutations in Japan in our own cohort and in an extensive review of the literature. Notably, six patients with double mutations of *PROC* developed no overt thromboembolic events until they reached 15 years of age. The genotypes and phenotypes of patients with neonatal- and late-onset thrombophilia are discussed.

Patients and Methods

Subjects and data collection

Clinical data were collected from our cohort genetic study and a literature review. Twelve patients were diagnosed with biallelic *PROC* defects, as assessed by the thrombophilic screening of plasma PC activity and/or a genetic study at Kyushu University from 1993 to 2015. This study was certified by the Institutional Review Board of Kyushu University (#232–02) and Yamaguchi University (H26-136). Written informed consent was obtained from all of the subjects. The collected data included gender, the age at the onset of each thromboembolic event, the family history of PC deficiency, PC antigen and activity levels, genetic study results, and the outcomes. We further reviewed all of the publications and sentinel sources, including meeting reports in Japan, using the Japana Centra Revuo Medicina, PubMed, and Google Scholar for citations that were published from 1981 to December 2015. The search terms were congenital, inherited, hereditary or heritable PC deficiency, compound heterozygous, PF and thrombophilia. We collected 22 PC-deficient patients for our analysis, including 12 from our cohort and 10 from the extensive review [13-19].

Coagulation study

Coagulation tests were performed in our cohort as described previously [4]. The anticoagulant activities of PC were determined using the Staclot Protein C kit (Diagnostica Stago, Asnieres, France). The

reference ranges of the PC activity/antigen levels for term and preterm infants and other subjects were based on those reported in previous studies [4,6,8,20]. In our cohort study, the plasma PC activity was determined using repeated coagulation tests (including factor VII activity) at the time of diagnosis and during the disease course.

Genetic analyses

Genomic DNA was extracted from peripheral blood leukocytes after obtaining informed consent from the patients. The direct sequencing of polymerase chain reaction (PCR) products was performed for the coding regions of *PROC* (exons 1–9), as described previously [6]. The exon and exon-intron boundary regions of each gene, including the promoter region, were amplified by PCR, and the products were then subjected to direct sequencing using an ABI 377 (Applied Biosystems, Foster City, CA, USA).

Results

Clinical presentation of PC-deficient patients with double mutations

The characteristics of all 22 patients who had double-allele *PROC* mutations are summarized in **Table 1**, based on our cohort and a review of the cases that were reported between 1985 and 2015 [4,6,9]. There was no declared consanguinity in the 20 unrelated families, including twins (Patients [Pts] 12-1 and 12-2) and a child (Pt 16-1) and his mother (Pt 16-2). They had 4 homozygous and 18 compound heterozygous mutations. Sixteen newborns first presented with either PF (n=11, 69%), intracranial thrombosis and hemorrhaging (ICTH) (n=13, 81%), or both (n=8, 50%). Of the 16 neonatal-onset patients, 2 had prenatal cerebral lesions that were assessed by imaging analyses, and 11 and 6 infants presented within 7 days and within the first 24 h after birth, respectively.

Six patients first presented after the neonatal period. Two developed deep vein thrombosis (DVT) in adolescence and early adulthood, while 2 others were elderly patients with cerebral infarctions. Pt 16-1 first presented with epilepsy at 3 years of age but did not experience overt thrombosis without anti-coagulant therapy until 12 years of age. Pt 17 was diagnosed with PC deficiency at 22 years of age, when a subclinical hypercoagulable state was identified during the persistent febrile period of cervicitis (high D-dimer levels and sustained low levels of plasma PC activity). The detailed clinical information of Pts 16-1, 16-2, and 17 in our cohort are shown in the **Supplemental Table S1**.

Plasma PC activity and the genotype of the neonatal- and late-onset cases

All but one of the neonatal onset patients (Pt 11) showed a plasma PC activity of <10% (the lowest detectable limit) at the time of their diagnosis. In contrast, the late-onset patients showed a median PC activity of 31% (range: 19-52%) at the time of their diagnosis. In Japan, 5 recurrent mutations (ex7: c.541T>G, p.F181V, ex7: c.631C>T, p.R211W [PC-Tochigi], ex9: c.1015G>A, p.V339M, ex9: c.1218G>A, p.M406I, ex9: c.1268delG, p.G423VfsX82 [PC-Nagoya]) were reportedly found in 49% of Japanese families with PC deficiency [19]. Fifteen of 22 patients (68%) had these 5 major mutations (p.G423VfsX82 [n=7], p.V339M [n=4], p.R211W [n=3], p.M406I [n=3], and p.F181V [n=71] or 2 other reported mutations (p.E68K [n=3], and p.K193del [PC-Tottori] in [n=73] in Japan. PC-Nagoya was the most frequent allele (22%; 7/32) in the neonatal onset cases (44%; 7 of 16 patients). In contrast, PC-Tottori was the most frequent allele (25%; 3/12) in the late-onset cases (50%; 3 of 6 patients). Two of the 5 major mutations in the neonatal cases (G423VfsX82, V339M) were not found in the late-onset cases, while the other 3 mutations in neonatal cases (R211W, M406I, and F181V) were found in late-onset cases. The novel mutation p.A309V was found in a late-onset family (Pts 16-1 and 16-2).

Discussion

The present study first characterized the onset of disease and the genotype of PC deficiency in patients with double mutations. Early-onset patients presented with PF and/or ICTH within 24 h after birth, with PC activity levels of <10%. Late-onset patients developed overt DVT at >15 years of age, with a PC activity of >20%. The genotypes of the neonatal cases were highly restricted to the major Japanese mutations [6,7,19]. In contrast, the late-onset patients showed genotypes that were distinct from the neonatal cases, including p.K193del and a novel mutation. The distinct mutation spectrum in patients of this ethnic background may explain the first presentation of PC-deficient patients with double mutations.

The first survey of double *PROC* mutations demonstrated that there were 2 types of disease: the majority of cases were classified as neonatal onset (72%), while in the remaining late-onset cases, thromboembolic events first occurred after adolescence. The present cohort corroborated the finding that the majority of PC-deficient patients with double *PROC* mutations first presented with neonatal PF and/or ICTH [9]. More than 200 *PROC* mutations have been reported. The high allele frequency of PC-Nagoya (44%) in the newborns was as we previously predicted [3,9]. The onset age and mode of the late-onset patients with double mutations were varied. Late-onset patients with the double mutations of *PROC* are rarely reported in Caucasians [21,22] but are increasing in number in Asian countries [18,19,23-32] (**Table 2**). This discrepancy may be due to the high allelic frequency of FV Leiden and FII variant in Caucasians, which hampers the detection of rare double *PROC* mutations in

adult patients. Manabe et al. [32] previously reported a late-onset case in a patient with genetically undetermined PC deficiency (<5% of PC activity) who first presented with DVT at 14 years of age. Although he was also found to have dysplasminogenemia, which might augment hypercoagulability, the late onset of the patient's disease was not explained. Iijima et al. [18] reported a patient with homozygous K193del as PC-Tottori, who first developed DVT at 23 years of age, with a non-severe PC activity level of 52% (**Table 1**, Pt 18). Recent large studies in Japanese adults have reported no patients having double mutations of *PROC* [6,7]. In contrast, Miyata et al. [19] previously recognized K193del as a polymorphism because the PC activity levels were subnormal, as shown in the 2 elderly patients with PC activity >60% (**Table 1**, Pt 19 and Pt 20). K193del has been recently identified as the most common variant in Chinese patients, although the amidolytic PC activity-based screening system does not discriminate the variant carrier from non-carrier [33]. K193del, M406I and R211W continue to be found in Korean [31] and Japanese patients with late-onset double *PROC* mutations (**Table 2**). In this line, the variant allele of K193del may be prevailing as a type II deficiency in Asian patients with late-onset double *PROC* mutations.

A late-onset family (Pt 16-1 and Pt 16-2) harbored a novel mutation of c.926C>T in exon 9. Pts 16-1 and 17 were diagnosed with moderately severe type I and type II PC-deficiency, respectively. The levels of PC activity in Pt 16-1 were >10% during infancy and >20% during his school life (*data not shown*). A PC activity level of 20% was the hallmark of the late-onset cases (**Table 1**). The disease onset of biallelic PC deficiency might depend on the genotype that retains 20% of the PC activity levels in daily life.

A major concern is the *true* prevalence of inherited PC deficiency. Pts 16 and 17 were found in our pediatric thrombophilia cohort [4,9]. The low allelic frequency of *PROC* mutations in patients of other ethnicities (<0.5%) was originally estimated from a population study based on the assessed plasma activity. A single assay of natural anticoagulants lacks the power necessary to determine the inherited deficiency. The asymptomatic parents with a heterozygous *PROC* mutation, such as F181V and K193del in this study (**Supplemental Table S1**) and our recent report [11], probably show a normal PC activity level. In pediatric screenings, physiologically low concentrations of natural coagulants also hamper reaching a diagnosis of inherited thrombophilia. Thus, the *true* frequency of Japanese carriers of a heterozygous *PROC* mutation may be higher than the previous reports. It is necessary to clarify the *true* frequency that we would find out clinical and laboratory signs in asymptomatic variants. Further studies should be directed towards the early diagnosis of asymptomatic PC deficiency in various conditions, especially for the late-onset type of double mutations.

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Legends

Supplemental Table S1. The clinical profiles of three patients with the late-onset type PC deficiency in our cohort

Pt, patient; DVT, deep vein thrombosis; PC, protein C; n.a, not available; rr, reference range; *PROC*, protein C-gene; c.hetero, compound heterozygote; homo, homozygote; PTE, pulmonary thromboembolism

*: The details of the *PROC* mutation are shown in **Table 1**.

Case presentations

Pt 16-1 and Pt 16-2

Pt 16-1 and Pt 16-2 were a 12-year-old boy and his 45-year-old mother (at the time of writing this manuscript). Pt 16-1 was born at 39 gestational weeks, weighing 3,752 g, by an elective caesarean delivery to a PC-deficient mother. The family history revealed thrombosis in the mother's but not father's relatives (Supplementary Figure 1A). None of the family members had received a genetic diagnosis. The mother (Pt 16-2) suffered from DVT and a pulmonary thromboembolism at 15 and 17 years of age, respectively. Despite the administration of warfarin therapy, she experienced recurrent abortions because of DVT at 20, 25 and 26 years of age. She gave birth to the boy (Pt 16-1) at 34 years of age after a pregnancy course that involved prolonged heparinization and the replacement of plasma-derived activated PC (Anact C®). Pt 16-1 lived an active neonatal life, with a PC activity of 13% at 5 days after birth (Supplementary Figure 1B). Thereafter, the PC activity levels were

approximately 20%. Partial epilepsy occurred at 3 years of age but subsided at 5 years of age. Magnetic resonance imaging and electroencephalography indicated no abnormalities. A physical examination revealed that the boy had no abnormalities, and he has continued to enjoy participating in a judo club after school. Coagulation studies revealed a normal prothrombin time and activated partial thromboplastin time. The plasma levels of protein induced by the absences of vitamin K or antagonist-II were undetectable. The fibrinogen concentration was below normal (165 mg/dL, reference range: 200-400 mg/dL), but none of the fibrinogen degradation products or D-dimer levels increased. The patient's thrombin-antithrombin complex, α2-plasmin inhibitor-plasmin complex, thrombomodulin, plasminogen activity, and plasminogen activator-tissue plasminogen activator inhibitor-1 complex levels were all normal. The patient's antithrombin (110%) and PS activity (73%) levels were normal for his age. The plasma PC activity (27%) and antigen (21%) levels were low for his age (Figure 1B). The patient was negative for lupus-anticoagulants. A genetic analysis using peripheral blood-derived DNA revealed a reported mutation of c.541T>G, p.F181V in exon 7 and an unreported one of c.926C>T, p.A309V in exon 9. His 49-year-old father, who had no thrombotic history, showed a PC activity of 80% (reference range: 75-131%) and carried a heterozygous mutation in exon 7 (c.541T>G). The exon 9 mutation of c.926C>T, but not the wild genotype, was found in the mother.

Pt 17

Pt 17 was a 22-year-old female patient who was referred to us because of a transient increase in her D-dimer levels during the febrile period of cervicitis. Subclinical coagulopathy subsided uneventfully without anti-coagulation therapy. She showed no abnormalities on physical and laboratory examinations. She had no personal or family history of illness. Coagulation studies revealed a normal prothrombin time and activated partial thromboplastin time. Protein induced by the absence of vitamin K or antagonist-II was undetectable. The patient's fibrinogen, fibrinogen degradation products, D-dimer, thrombin-antithrombin complex, α2-plasmin inhibitor-plasmin complex, thrombomodulin, plasminogen, and plasminogen activator-tissue plasminogen activator inhibitor-1 complex levels were all normal. The patient's plasma PC activity (17%) and antigen levels (50%) but not antithrombin (99%) or PS activity (78%) were low. The patient was negative for lupus-anticoagulant. A genetic analysis revealed compound heterozygous *PROC* mutations of c.577-579delAAG, p.K193del in exon 7, and c.631C>T, p.R211W in exon 7. A family study was not performed because informed consent could not be obtained.