Received: 24 June 2016 | Revised: 15 November 2016 | Accepted: 15 November 2016

DOI: 10.1002/pbc.26404

RESEARCH ARTICLE

WILEY Pediatric Blood & Cancer

SIOP INTERNATIONAL SOCIETY OF PAEDIATRIC ONCOLOGY

aspho The American Society of Pediatric Hematology/Oncology

The clinical presentation and genotype of protein C deficiency with double mutations of the protein C gene

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Grant sponsor: Ministry of Health, Labor and Welfare of Japan.

[This article was modified in May 2017 after initial online publication. The mutation of patient number 20 in Table 1, "ex8: c.730C>T,p.H244W/ex9: c1203G>A,p.D401N" was corrected to "ex8: c.730C>T,p.H244W/ex9: c1201G>A,p.D401N".]

Abstract

Background: Severe protein C (PC) deficiency is a rare heritable thrombophilia leading to thromboembolic events during the neonatal period. It remains unclear how individuals with complete PC gene (*PROC*) defects develop or escape neonatal stroke or purpura fulminans (PF).

Procedure: We studied the onset of 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 PF (n = 11, 69%), intracranial thromboembolism and hemorrhage (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 five major mutations (G423VfsX82, V339M, R211W, M406I, and F181V) or two others (E68K and K193del) that have been reported in Japan. Three of the six 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 family of two patients with late onset.

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).

KEYWORDS

compound heterozygous mutation, pediatric stroke, pediatric thrombophilia, protein C deficiency, purpura fulminans

1 | 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. The natural anticoagulant deficiencies of PC, PS, and antithrombin are thus the leading genetic causes 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 PC deficiency, while 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

Abbreviations: DVT, deep vein thrombosis; FII variant, prothrombin G20210A; FV Leiden, factor V G1691A; ICTH, intracranial thrombosis and hemorrhage; PC, protein C; PCR, polymerase chain reaction; PF, purpura fulminans; *PROC*, PC gene; PS, protein S; Rr, reference range

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.

2 | PATIENTS AND METHODS

2.1 | 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]

2.2 | 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 (Rr) 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.

2.3 | 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).

3 | RESULTS

3.1 | 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 12-1 and 12-2) and a child (Patient 16-1) and his mother (Patient 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 hr after birth, respectively.

Six patients first presented after the neonatal period. Two developed deep vein thrombosis (DVT) in adolescence and early adulthood, while two others were elderly patients with cerebral infarctions. Patient 16-1 first presented with epilepsy at 3 years of age but did not experience overt thrombosis without anticoagulant therapy until 12 years of age. Patient 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 Patients 16-1, 16-2, and 17 in our cohort are shown in the Supplementary Table S1.

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

All but one of the neonatal onset patients (Patient 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, five 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 five major mutations (p.G423VfsX82 [n = 7], p.V339M [n = 4], p.R211W [n = 3], p.M406I [n = 3], and p.F181V [n = 71] or two 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%; three of six patients). Two of the five

TABLE 1 The disease onset and the genotype of Japanese patients with double mutations of the protein C gene

Patient no.	Sex	Age onset	Diagnosis	PC activity (%)	Mutation	Estimated origin	Familial VTE	Reference
1	F	GA33w	Hydrocephaly, PF	<10	ex9: c.1141G>A, p.V381M	m/f	Nonconsang.	OC[4,9]
2	M	Fetus	ICTH, PF	<5	ex3: c.142G>A, p.E48K/ **ex3: c.202G>A, p.E68K**	m/f	No	OC
3	F	0 d	ICTH, PF	3	**ex9: c.1268delG, p.G423VfsX82**[a]	m/f	Nonconsang.	13
4	F	0 d	ICTH, PF	<10	**ex9: c.1015G>A, p.V339M/ ex9: c.1268delG, p.G423VfsX82**[a]	m/f	No	OC[4,9]
5	M	0 d	ICTH	<10	ex8: c.688-690delCTG, p.L230del/ **ex9: c.1015G>A, p.V339M**	m/f	No	OC
6	M	0 d	PF	<5	**ex7: c.631C>T, p.R211W**[b]**/ ex9: c.1268delG, p.G423VfsX82**[a]	m/f	No	OC[4,9]
7	F	0 d	PF	<10	ex3: c.124C>A, p.R42S/ **ex9: c.1218G>A, p.M406I**	m/f	No	14
8	M	0 d	PF	10	**ex7: c.631C>T, p.R211W**[b]**/ ex9: c.1268delG, p.G423VfsX82**[a]	m/f	No	14
9	M	1 d	ICTH, PF	5	**ex3: c.202G>A, p.E68K/ ex9: c.1268delG, p.G423VfsX82**[a]	m/f	nd	15
10	M	2 d	ICTH, PF	<10	**ex9: c.1015G>A, p.V339M**/ ex9: c.1003C>T, p.Q335X	m/f	No	16
11	F	4 d	ICTH, PF	17	**ex3: c.202G>A, p.E68K/ ex9: c.1015G>A, p.V339M**	m/f	No	OC
12-1	F	6 d	ICTH	<5	ex8: c.793C>T, p.L265F/ ex9: c.1266G>C, p.W422C	m/f	No	OC[4,9]
12-2	F	6 d	ICTH	<5	ex8: c.793C>T, p.L265F/ ex9: c.1266G>C, p.W422C	m/f	No	OC[4,9]
13	F	13 d	ICTH	<5	ex4: c.164delT, p.L55RfsX6/ ex9: c.811C>T, p.R271W	m/f	No	17
14	M	Neonate	ICTH, PF, DVT	<5	**ex9: c.1268delG, p.G423VfsX82**[a]	nd	Nonconsang.	OC
15	nd	Neonate	ICTH	6	**ex9: c.1218G>A, p.M406I/ ex9: c.1268delG, p.G423VfsX82**[a]	m/f	nd	19
16-1	M	3 y	*Epilepsy*	27	**ex7: c.541T>G, p.F181V**/ ex9: c.926C>T, p.A309V[c]	m/f	yes	OC
16-2	F	15 y	DVT, PTE	nd	ex9: c.926C>T, p.A309V[c]	m/f	Nonconsang.	OC
17	F	22 y	*Coagulopathy*	19	**ex7: c.577-579delAAG, p.K193del/ ex7: c.631C>T, p.R211W**[b]	nd	No	OC
18	M	23 y	DVT	52	**ex7: c.577-579delAAG, p.K193del**	m/f	No	18
19	nd	61 y	ICTH	33	**ex7: c.577-579delAAG, p.K193del/ ex9: c.1218G>A, p.M406I**	nd	nd	19
20	nd	63 y	ICTH	31	ex8: c.730C>T, p.H244W/ ex9: c.1201G>A, p.D401N	nd	nd	19

The bold (recurrent) and underlined mutations are the major five mutations in Japanese protein C deficiency; these include PC-Nagoya ([a]) and PC-Tochigi ([b]).[19] The bold 3-base deletion in ex7 (c.577-579delAAG, p.K193del, PC-Tottori) was reported as the most common mutation in Chinese thrombophilia.[33] The italic diagnoses indicate the first presentations suggesting thromboembolism. ICTH, intracranial thrombosis and hemorrhage; PF, purpura fulminans; DVT, deep vein thrombosis; PTE, pulmonary thromboembolism; GA, gestational age; M, male; F, female; w, week; d, day; y, year; m, mother; f, father; ex, exon; nd, not described/determined; consang, consanguinity; OC, our cohort.

[c]A novel mutation.

TABLE 2 The reported cases of late-onset thrombophilia in Caucasians and Asians with double *PROC* mutations

Patient no.	Sex	Age at onset	First presentation	Plasma PC activity (%)		Mutation	Family history of VTE	Nation	Reference
Caucasian									
1	F	16	DVT	8		p.A267T	No	Norway	21
2	F	18	DVT		3–13	p.F130AfsX, p.F181L	Yes	Austria	22
Asian									
1-1	M	10	DVT	10		**p.F181V**	Sister, cons.	China	23
1-2	F	43	DVT	20		**p.F181V**	Brother, cons.	China	23
2-1	M	15	DVT	4		**p.D297H**, p.V420L	Sister	China	24, 25
2-2	F	20	DVT	5		**p.D297H**, p.V420L	Brother	China	24
3	nd	24	DVT, PTE	10		p.F118Afs16, **p.F181V**	nd	China	25
4	F	30	Sinus thrombosis	18		**p.F181V**, **p.D297H**	Yes	China	26, 23
5	M	38	DVT	34		**p.F181V**, p.R199X,	nd	China	27
6	nd	46	DVT, PTE	58		**p.D297H**	nd	China	25
7	nd	42	DVT, stroke	39		**p.F181V**, p.E327V	nd	China	25
8	nd	14	DVT	16		p.R273H, **p.R290W**, p.D297H	nd	Taiwan	28
9	nd	41	DVT	60		**p.K193del**, **p.R290W**	nd	Taiwan	28
10	nd	44	DVT	37		p.V206A, **p.R290W**	nd	Taiwan	28
11	M	4	DVT, epilepsy	32		**p.R290W**	nd	Thai	29
12	M	19	DVT	4		p.P210L, **p.R211W**	Yes	Korea	30, 31
13	M	22	Budd-Chiari	2		**p.D297H**, **p.M406I**	No	Korea	30, 31
14	M	33	PTE	47		**p.K193del**, **p.M406I**	nd	Korea	31
15	F	57	DVT	41		p.R189W, **p.K193del**	nd	Korea	31
16	F	24	DVT	79		p.A301T, **p.M406I**	nd	Korea	31
17	F	56	DVT	62		**p.K193del**, p.G239W	nd	Korea	31
18	M	14	DVT	<5			Yes	Japan	32
19-1	M	*12*	*Epilepsy*[a]	27		**p.F181V**, p.A309V	Mother	Japan	Our cohort
20-2	F	15	DVT, PTE	nd		p.A309V	Yes	Japan	Our cohort
21	F	22	*Coagulopathy*[a]	19		**p.K193del**, **p.R211W**	nd	Japan	Our cohort
22	M	*23*	DVT	52		**p.K193del**	No	Japan	18
23	nd	61	Stroke	33		**p.K193del, p.M406I**	nd	Japan	19
24	nd	63	Stroke	31		p.H244W, p.D401N	nd	Japan	19

Bold indicates recurrent mutations. DVT, deep vein thrombosis; PTE, pulmonary thromboembolism; nd, not described; cons., consanguinity.
[a]Patients 16-1 and 17 had no imaging analysis verified thrombosis until 12 and 23 years of age, respectively.

major mutations in the neonatal cases (G423VfsX82, V339M) were not found in the late-onset cases, while the other three 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 (Patients 16-1 and 16-2).

4 | 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 hr 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 two 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 nonsevere PC activity level of 52% (Table 1, Patient 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 two elderly patients with PC activity >60% (Table 1, Patients 19 and 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 noncarrier.[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 (Patients 16-1 and 16-2) harbored a novel mutation of c.926C>T in exon 9. Patients 16-1 and 17 were diagnosed with moderately severe type I and type II PC deficiency, respectively. The levels of PC activity in Patient 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. Patients 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 (Supplementary 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 toward the early diagnosis of asymptomatic PC deficiency in various conditions, especially for the late-onset type of double mutations.

ACKNOWLEDGMENTS

We thank Dr. Brian Quinn (Editor-in-Chief of Japan Medical Communication) for editing the manuscript. This work was supported by Grants-in-Aid for Scientific Research (Research on Intractable Diseases) from the Ministry of Health, Labor and Welfare of Japan.

AUTHOR CONTRIBUTIONS

The contributions of each author are as follows. HI and SO were the principal investigators, taking primary responsibility for the paper. ST, TS, MI, and TH performed the clinical management with helpful discussion regarding the completion of the work. MU completed the genetic analysis. YK, TH, and TU managed the quality control of the screening of thrombophilic predisposition. SO and DK organized the nationwide cohort of pediatric thrombophilia in Japan.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Inoue H, Terachi S-i, Uchiumi T, Sato T, Urata M, Ishimura M, Koga Y, Hotta T, Hara T, Kang D, Ohga S. The Clinical presentation and genotype of protein C deficiency with double mutations of the protein C gene. *Pediatr Blood Cancer*. 2017;64:e26404. DOI: 10.1002/pbc.26404