American Heart Journal Plus: Cardiology Research and Practice 50 (2025) 100496

Contents lists available at ScienceDirect

American Heart Journal Plus: Cardiology Research and Practice

journal homepage: www.sciencedirect.com/journal/american-heart-journal-plus-cardiology-research-and-practice

Research paper

Protein C deficiency with recurrent systemic thrombosis associated with compound heterozygous *PROC* missense variants

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ARTICLE INFO

Keywords:
Thrombosis
Inherited protein C deficiency
Exome sequencing

ABSTRACT

Herein, we identified compound heterozygous *PROC* missense variants in a protein C deficient patient with recurrent thrombotic events, including intestinal necrosis, extrahepatic portal vein obstruction, and lower limb venous thrombosis. The patient's protein C activity and antigen levels were extremely low (<10 % and 5 %, respectively). Exome sequencing analysis revealed two rare missense variants (c.76G>A:p.Val26Met in exon 3 and c.1000G>A:p.Gly334Ser in exon 9), both confirmed to be associated with protein C deficiency and one synonymous variant (c.423G>T:p.Ser141Ser in exon 6) in *PROC*. PCR amplification of genomic DNA spanning these exons followed by Sanger sequencing analysis revealed that the c.76G>A and the synonymous c.423G>T variants were in the same allele, whereas the c.1000G>A variant was on the opposite allele, indicating compound heterozygosity. Western blot analysis of Huh-7 and HEK293T cells transfected with expression vectors encoding *PROC* with or without these variants demonstrated that Gly334Ser-PROC expression levels were significantly decreased in culture media collected from HEK293T cells, while the expression levels of protein C with these variants were not significantly altered in cell lysates. This suggests that these variants may affect both protein activity and the secretory process of protein C.

1. Introduction

The prevalence of inherited protein C deficiency (PCD) in the general population is approximately 0.2–0.4 % [1,2]. Patients most commonly present with heterozygous PCD, whereas homozygous PCD is rare, occurring in 1 of 500,000–750,000 live births [3]. Homozygous individuals may develop life-threatening purpura fulminans due to severe thrombosis and embolism immediately after birth [4]. PCD is caused by various genetic variants including missense, nonsense, splice site, deletions, and insertions variants. Among them, missense variants throughout the coding region of *PROC* are the most prevalent [5]. Herein, we describe a case of a patient with lower-extremity thrombophlebitis, followed by congestive leg ulcers and mesenteric arterial embolism, leading to small-intestine necrosis. Genetic analysis combined with functional analysis demonstrated that the combined molecular dysfunctions caused by compound heterozygous variants may underlie the clinical course of this patient showing late-onset PCD. This case highlights the necessity for early diagnosis and attention to the recurrence of possible congenital deficiencies or abnormalities in anticoagulation and fibrinolysis factors. Recent studies have focused on congenital thrombotic diseases associated with deficiencies or molecular abnormalities of protein C, protein S, antithrombin, and plasminogen. PCD has been examined at the genetic, molecular, and cellular levels [6,7]. A detailed understanding of the molecular and genetic bases of PCD has significant implications for patient care to facilitate personalized treatment approaches and better management of thrombotic risks.

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https://doi.org/10.1016/j.ahjo.2024.100496
Received 19 August 2024; Received in revised form 9 November 2024; Accepted 11 December 2024
Available online 18 December 2024
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2. Materials and methods

2.1. Ethics

The use of human samples and genomic analysis was approved by the Ethics Committees of Osaka Police Hospital (No. 1998) and Osaka University Hospital (No. 684-8). Written informed consent was obtained from the patient at Osaka Police Hospital and Osaka University Hospital. The study adheres to the Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan, as well as all principles outlined in the Helsinki Declaration of 1964 and its subsequent amendments.

2.2. DNA sequencing

Genomic DNA was extracted from the patient's peripheral blood using the QIAamp DNA Mini Kit (QIAGEN) and subjected to whole-exome sequencing analysis. Genomic regions including c.76G>A (exon 3), c.423G>T (exon 6), and c.1000G>A (exon 9) were amplified by PCR using the following primer pairs (5′-3′): AGAGGACCCCTGCGCCAAGCCAT and GCTGCCCCAAGGCTCAACTC; and GTGCTTGGTCTTGCCCTTGGAGCA and ACACAGCATGTTCTCAGACACCA. PCR (KOD Fx Neo, TOYOBO) conditions were as follows: 94 °C for 2 min, followed by 33 cycles of 98 °C for 10 s, 72 °C for 30 s, and 68 °C for 180 s. PCR products were cloned into the cloning vector (pCR bluntII, Thermo) and analyzed by Sanger sequencing.

2.3. Cell culture and transfection

Human hepatoma Huh7 cells or HEK293T cells were seeded in 6-well plates at a density of 2×10^5 cells per well in Dulbecco's Modified Eagle Medium (DMEM). The coding sequence of the human *PROC* gene (NM_000312) was synthesized by Integrated DNA Technologies (IDT) and cloned into an expression vector under the control of the human cytomegalovirus promoter with a 3′ terminal HA tag. c.76G>A and c.1000G>A variants were inserted using inverse PCR. Expression vectors encoding wild-type *PROC* (WT-PROC) and two variants (Val26Met- and Gly334Ser-PROC) were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. Protein C activities of lysate and culture media were measured by SRL, Inc., Japan.

2.4. Western blotting

Forty-eight hours post-transfection, cell lysates, and culture media were collected; their protein concentrations were measured using the BCA Protein Assay Kit. Samples (10 μg from cell lysates and 40 μg from culture media) were loaded onto 10 % gels and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 45 min, followed by protein transfer to membranes at 15 V for 30 min. Membranes were blocked with 3 % non-fat dry milk in TBS-T (Tris-buffered saline with 0.1 % Tween-20) for 30 min and incubated overnight at 4 °C with primary antibodies against protein C (ProteinTech, 25382-1-AP) and GAPDH (Santa Cruz Biotechnology, sc-47724). After washing with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were imaged using the ChemiDoc Touch MP Imaging System (Bio-Rad).

2.5. Statistical analyses

All analyses were conducted using JMP software (version 17.0.0; SAS Institute Inc.). Graphs were plotted using the GraphPad Prism software (version 9.1.1; GraphPad Software, Inc.). The Shapiro–Wilk test was used to assess data normality. Quantified levels of protein C that were not normally distributed were analyzed using the Kruskal–Wallis test followed by Dunn's test. All data are expressed as medians with 25th and 75th percentiles. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Patient with recurrent systemic thrombosis

A 50-year-old female patient presented with recurrent systemic thrombosis (Fig. 1A). There was no significant family history of thrombosis. Her father died of cancer in early middle age, and her mother had cirrhosis. Her medical history included lower-limb thrombophlebitis at 15 years of age, post-thrombotic leg ulcers 11 years later (Fig. 1B), and intestinal necrosis due to superior mesenteric artery thromboembolism at age 38 that necessitated an urgent life-saving surgery. She survived and was eventually discharged from the hospital. Following these events, blood tests revealed complete PCD. Since then, she has been undergoing treatment with warfarin anticoagulation therapy. At 40 years of age, she developed esophageal variceal bleeding due to extrahepatic portal vein obstruction (Fig. 1C). At her current age of 50, we continue to manage recurrent thrombophlebitis with outpatient follow-up. Despite achieving a target PT-INR of 2.0–2.5, she continues to experience recurrent ulcers associated with lower-limb venous thrombosis.

3.2. Laboratory data

The patient's blood was sampled at the age of 50 while she was taking a daily dose of 10 mg of warfarin. Protein C levels were below the detectable threshold. The extremely low activity (<10 %) and antigen levels (<5 %) (Table 1) prompted genetic analysis.

3.3. Genetic analyses

Genomic DNA was extracted from the patient's peripheral blood and subjected to whole-exome sequencing analysis. Two missense variants (c.76G>A:p.Val26Met and c.1000G>A:p.Gly334Ser) and one synonymous variant (c.423G>T:p.Ser141Ser) in *PROC* (NM_000312) were identified. p.Val26Met and p.Gly334Ser are rare, with allele frequencies of 0.000001239 and 0.000007436 in the gnomAD database [8], respectively. p.Val26Met is likely pathogenic [9], while p.Gly334Ser has conflicting interpretations [8], although homozygosity for the p.Gly334Ser (also termed p.Gly292Ser) substitution has been identified in a Japanese patient with recurrent thromboembolism and complete PCD [10]. Because genomic information from the patient's parents was unavailable, we sought to determine the allele location of each variant by genomic PCR analysis. The identified synonymous variant c.423G>T (p.Ser141Ser) was in exon 6, between the two missense variants c.76G>A (p.Val26Met) and c.1000G>A (p.Gly334Ser) in exons 3 and 4,

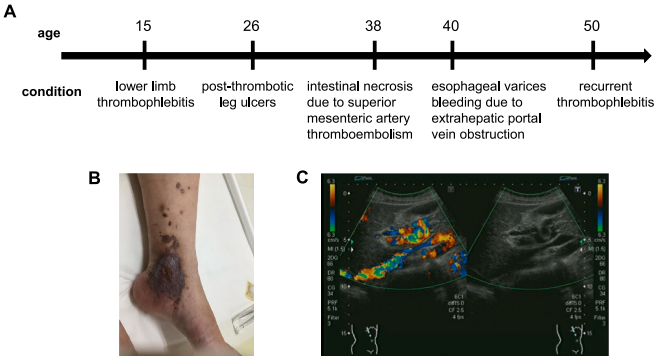

Fig. 1. Clinical progression.
(A) Timeline. (B) Lower-limb thrombophlebitis and (C) Abdominal ultrasound examination of the extrahepatic portal vein obstruction.

Table 1
Laboratory data.

Parameter	Value	Parameter	Value
White blood cells	2.40 × 10^3/μL	PT (%)	26 %
Red blood cells	4.41 × 10^4/μL	PT (seconds)	30.6 s
Hemoglobin	12.8 g/dL	PT (ratio)	2.34
Hematocrit	38.6 %	PT (INR)	2.55
Platelets	113 × 10^4/μL	APTT (seconds)	48.5 s
Fibrinogen	203 mg/dL	APTT (ratio)	1.894
D-dimer	3.72 μg/mL	Protein C antigen	0 %
Anti-cardiolipin β2 glycoprotein I	≤1.2 U/mL	Protein C activity	<10 %
Anti-cardiolipin antibody IgG(E)	<4.0 U/mL	Protein S (antigen) LA method	43 %
Factor VIII activity	226 %	Protein S (free antigen) LA method	26 %

PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; LA, lupus anticoagulant.

respectively (Fig. 2A). Sanger sequencing demonstrated that c.76G>A (p.Val26Met) and c.423G>T (p.Ser141Ser) were located on the same allele, whereas c.1000G>A (p.Gly334Ser) and c.423G>T (p.Ser141Ser) were on the other (Fig. 2B), indicating compound heterozygosity.

3.4. Expression of variant protein C

Cell lysates and culture media were collected from Huh7 cells or HEK293T cells transfected with the expression vector encoding human wild-type *PROC* (WT-PROC) or *PROC* carrying c.76G>A or c.1000G>A variants (Val26Met- or Gly334Ser-PROC) to evaluate whether the identified genetic variants affected the expression levels of protein C. Western blot analysis demonstrated that protein expression levels were not significantly altered among WT, Val26Met- or Gly334Ser-PROC in either cell lysates or culture media in Huh7 cells (Fig. 3A and B). However, protein expression levels of Gly334Ser-PROC collected from culture media in HEK293T cells were significantly decreased compared to WT, while expression levels of Val26Met- and Gly334Ser-PROC in cell lysates were comparable to those of WT-PROC (Fig. S1 and Fig. 3C and D). These data are consistent with the previous findings demonstrating that expression levels of Gly334Ser-PROC are significantly decreased in culture media collected from the transfected COS 7 cells, suggesting the impairment of the secretory process [10]. Additionally, we corrected both lysate and culture media samples after transfection with the expression vectors encoding WT-, Val26Met- or Gly334Ser-PROC, and measured protein C activities. However, protein C activities in all the samples were below measurement sensitivity (10 %), probably due to the lack of other circulating coagulation factors or other physiological modifications required for *in vivo* protein C activity usually included in clinical human plasma samples [11].

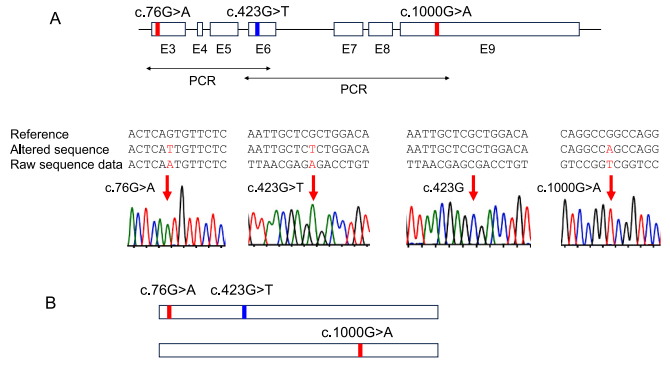

Fig. 2. Genetic analysis using PCR followed by Sanger sequencing analysis. (A) Schema of the exon 3 (E3) to exon 9 (E9) of the human *PROC* gene. The locations of the identified variants are highlighted in red (c.76G>A and c.1000G>A) and blue (c.423G>T). Targeted genomic regions for PCR analysis are indicated by arrows. The results of Sanger sequencing analysis using each PCR product are shown. (B) Approximate allele location of each variant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Protein C is a vitamin K-dependent serine protease precursor produced in the liver. It is among the most important physiological regulators of blood coagulation [12]. Thrombin, generated by the coagulation cascade, binds to thrombomodulin receptors on endothelial cell walls, further rapidly and specifically activating protein C [13]. Activated protein C degrades and inactivates Factors V and VIII, thereby playing an essential role in controlling intravascular coagulation [13,14]. Despite being heterozygous, patients with congenital protein C deficiency (PCD) typically experience severe venous thrombosis and embolism starting in adolescence [15,16]. More severe forms of PCD, including homozygous or compound heterozygous variants, occur at a frequency of 1 in 500,000–750,000 individuals. The incidence of asymptomatic PCD is between 1 in 200 and 1 in 500 healthy individuals [9].

We encountered a case of severe PCD that was severely recurrent in this study. Our patient experienced a wide range of systemic thrombotic events, from mild thrombosis to life-threatening superior mesenteric artery occlusion and intestinal necrosis. We identified compound heterozygous missense variants c.76G>A (p.Val26Met) and c.1000G>A (p.Gly334Ser) in this patient, which have been implicated in pathogenicity and appear to play a significant role in the observed clinical manifestations.

Homozygous p.Gly292Ser, the same mutation as p.Gly334Ser, causes recurrent thromboembolism and complete PCD [10]. Gly334Ser-PROC collected from culture media in HEK293T cells were significantly decreased compared to WT, suggesting the impairment of the secretory process. However, the detailed mechanism by which p.Gly334Ser causes disease remains unclear and is a subject for future research. The splicing analysis using HEK293T cells transfected by plasmid vectors demonstrates that c.76G>A (p.Val26Met) variant significantly increased pre-mRNA aberrant splicing, resulting in the changed open-reading frame of *PROC* [5]. The two different molecular dysfunctions consisting of splicing abnormality caused by c.76G>A (p.Val26Met) variant and impaired secretory process caused by c.1000G>A (p.Gly334Ser) variant may underlie the clinical course of this patient with late-onset PCD.

In heterozygous PCD, depending on the type of *PROC* variant, the decrease in protein C activity may be mild, and some cases may remain asymptomatic until adulthood. However, approximately 50 % of patients develop thrombosis by the age of 30 to 40 [17]. Additionally, PCD has been noted as an important risk factor for ischemic stroke and myocardial infarction [18]. By contrast, in homozygous and compound heterozygous PCD, severe and fatal fulminant purpura may occur during the neonatal period. This condition is characterized by microvascular thrombosis, cerebral thrombosis, and necrotic skin lesions due to disseminated intravascular coagulation [17]. The patient in this case had compound heterozygosity and exhibited a very severe phenotype. Notably, the onset occurred relatively late, as opposed to during the fetal

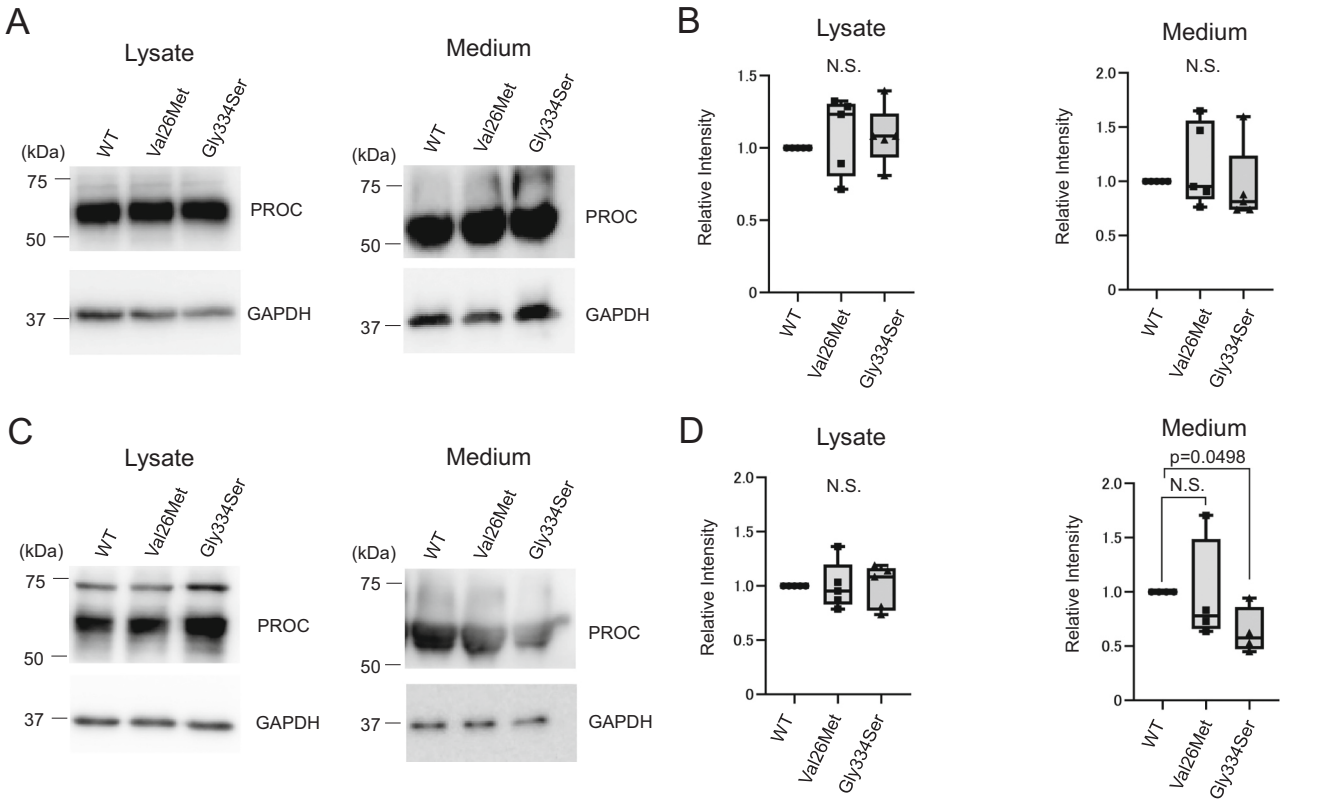

Fig. 3. Western blot analysis of the expression levels of protein C in Huh7 and HEK293T cells.
Hepatoma Huh7 cells (A) and HEK293T cells (C) were transfected by the expression vector encoding human WT-, Val26Met- or Gly334Ser-PROC. Forty-eight hours post-transfection, both the cell lysates and culture media were collected and analyzed by western blot using the indicated antibodies. Quantified protein expression levels normalized by GAPDH expression are shown for Huh7 cells (B) and HEK293T cells (D) (data are expressed as median with the 25th and 75th percentiles, Kruskal–Wallis test followed by Dunn's test, $n = 5$). N.S.: not significant.

or early childhood periods, as reported for these mutations. The identification of the p.Val26Met and p.Gly334Ser variants may aid in genetic counseling and early diagnosis of PCD. Genetic screening for these mutations in families can help assess the risk of thrombotic events. The interprofessional team-based approach including genetic counseling should be offered to at-risk patients with a family history of the disease [19]. Because the compound heterozygous variants in *PROC* identified in this patient were considered to be pathogenic, both genetic testing and genetic counseling should be considered for the PCD patient without significant family history.

Recent studies have reported the effectiveness of direct oral anticoagulants (DOACs) [20] as an alternative to warfarin, and in cases like this one, where it is difficult to control INR and a high dose of warfarin is required, DOACs may serve as a suitable alternative.

In the present study, we primarily evaluated protein expression in cultured cells. *In vivo* studies are required to better understand the functional effects of these variants at the molecular level, particularly those focusing on structure and activity. Previous reports have also recognized the use of transgenic mice for protein C, and it has been suggested that low protein C expression induces inflammation and thrombosis [21]. In the future, it is probable that genetically modified mice will be necessary for analysis. Understanding the mechanisms of aberrant splicing and exploring targeted therapies to correct these genetic defects is crucial.

5. Conclusion

Our results indicate a correlation between severe PCD, recurrent thrombotic events, and the identified compound heterozygous variants c.76G>A (p.Val26Met) and c.1000G>A (p.Gly334Ser). Gly334Ser-PROC expression levels were significantly decreased in culture media collected from HEK293T cells, while the expression levels of protein C with these variants were not significantly altered in cell lysates. This suggests that these variants may affect both protein activity and the secretory process of protein C. Understanding these mechanisms is essential for developing personalized treatment approaches and improving the management of patients with PCD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahjo.2024.100496.

CRediT authorship contribution statement

Mikio Shiba: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shuichiro Higo:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Yu Morishita:** Investigation, Data curation. **Yasuhiro Ichibori:** Investigation, Formal analysis, Data curation. **Yoshihiro Kin:** Writing – review & editing, Supervision. **Yasushi Sakata:** Writing – review & editing, Supervision. **Yoshiharu Higuchi:** Writing – review & editing, Supervision.

Funding

This study was supported by JSPS KAKENHI grant 24K02446.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Center for Medical Innovation and Translational Research and the Center for Medical Research and Education at the Graduate School of Medicine, Osaka University.

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