GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

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

L.-H. Yang[1], M.-S. Wang[1], F.-X. Zheng[1], J. Li [1], Y. Chen[2], Y.-H. Jin[1], H.-X. Xie[1], X.-L. Yang[1] and B.-C. Chen[3]

[1]Clinical Laboratory Diagnostic Centre,
The First Affiliated Hospital of Wenzhou Medical College, Wenzhou, China
[2]Department of Haematology,
The First Affiliated Hospital of Wenzhou Medical College, Wenzhou, China
[3]Wenzhou Key Laboratory of Surgery; Department of Surgery,
The First Affiliated Hospital, Wenzhou Medical College, Wenzhou, China

Corresponding author: B.-C. Chen
E-mail: chenbicheng@hotmail.com

Genet. Mol. Res. 13 (2): 2969-2977 (2014)
Received March 26, 2013
Accepted September 2, 2013
Published April 16, 2014
DOI http://dx.doi.org/10.4238/2014.April.16.5

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).

Table 1. Sequences of primers for 9 exons of PROC.

Exons	Primers sequence (5'→3')	PCR production (bp)	Tm (°C)
E1	GCTGAGCTAGGACCAGGAGTG CAAAGGGACCTGAGACTGTGG	346	57
E2	TGCTTTCTAGGCAGGCAGTGT GGAGGGAGCTTTAGGAGGTCA	552	58
E3	CATCTCAGAGCAAGGCTTCGT CTCCTAAGAGGGCCTCAGCAT	496	58
E4+5+6	TCGGGCGTCGATCCCTGTTTG CCGCTGCCCCAAGGCTCAACT	761	68
E7	CAGGAGGGCAGTCTCGGGAGGA CCCTGAGCATAGCTGCCAGGATGG	281	68
E8	ATGCCCATATGACCAGGGAAC GGGAGTGGAGAGGTGAAGGTC	516	58
E9a	GTCACCGTTGATAGGGTTCCA CGAGGTAGCGGCTGACTTTG	732	59
E9b	GGGCTCCTTCACAACTACGG GTCAAGCCTCACCTTCAGCA	475	58

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. The

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*.

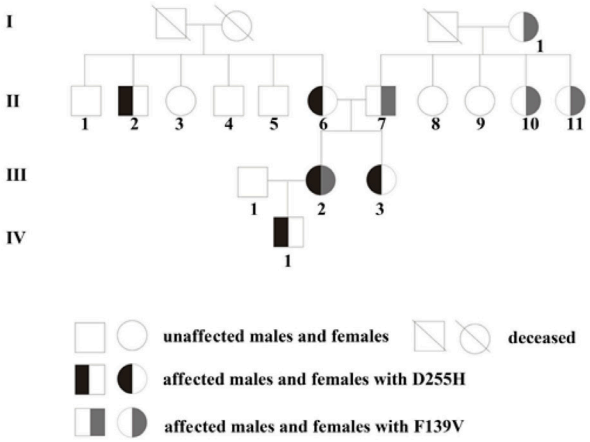

Figure 1. Pedigree of the family.

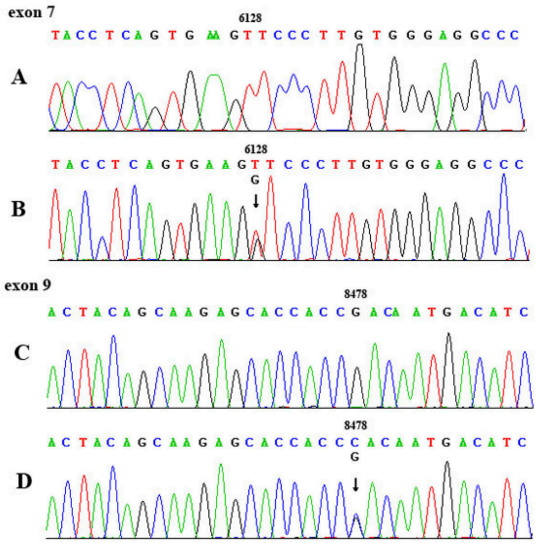

Figure 2. Mutations of PROC in proband with compound heterozygous protein C deficiency. The mutations of PROC were detected by sequencing. T6128G (*F139V*) in exon 7 and G8478C (*D255H*) in exon 9 were identified in a proband with compound heterozygous protein C deficiency (arrow).

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.

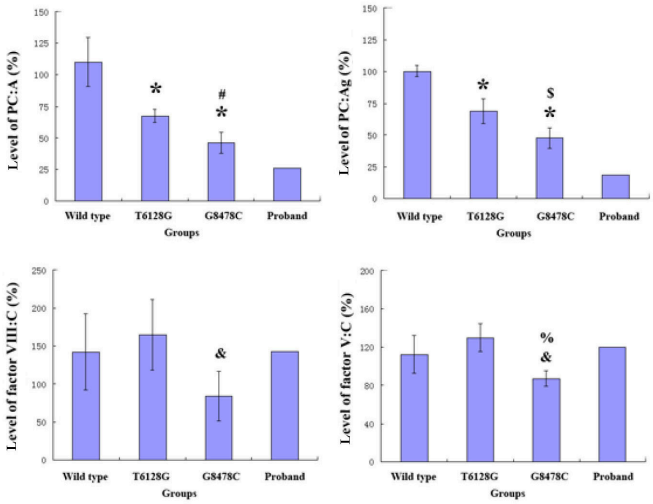

Figure 3. Coagulation tests of family members, family members carrying the heterogeneous mutation *F139V* or *D255H* have lower levels of PC:A and PC:Ag. *D255H* family members showed more significant impact on PC:Ag, factors V:C and VIII:C, compared to individuals with *F139V*. *P < 0.001, *vs* wild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

Family members	Age (years)	Disease	PC:A (%)	PC:Ag (%)	PS:A (%)	AT:A (%)	*F139V*	*D255H*
I_1	88	N	66	62.25	72	96	+	-
II_1	75	Hypertension	101	96.60	79	100	-	-
II_2	72	VTE	43	49.55	74	92	-	+
II_3	65	N	141	107.70	97	92	-	-
II_4	64	N	96	99.20	92	88	-	-
II_5	59	N	114	102.55	86	88	-	-
II_6	55	N	58	52.10	91	102	-	+
II_7	60	N	68	71.20	104	101	+	-
II_8	63	Type II diabetes	88	98.95	64	87	-	-
II_9	56	N	121	95.75	84	96	-	-
II_{10}	51	N	74	81.15	107	90	+	-
II_{11}	48	N	61	59.75	80	101	+	-
III_1	30	SSST	26	18.60	78	90	+	+
III_2	21	N	46	52.80	105	100	-	+
IV_1	5	N	38	35.50	69	88	-	+
Normal range			70-140	70-130	80-110	80-120	-	-

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 thromboembotic 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.

ACKNOWLEDGMENTS

Research supported by the Zhejiang Provincial Top Key Discipline and Technology Foundation of Zhejiang Province (#2011C37003).

REFERENCES

Aiach M and Gandrille S (1996). Molecular basis for protein C hereditary deficiency. *Haemostasis* (Suppl 26): 4: 9-19.

Berdeaux DH, Abshire TC and Marlar RA (1993). Dysfunctional protein C deficiency (type II). A report of 11 cases in 3 American families and review of the literature. *Am. J. Clin. Pathol.* 99: 677-686.

Deguchi K, Tsukada T, Iwasaki E, Wada H, et al. (1992). Late-onset homozygous protein C deficiency manifesting cerebral infarction as the first symptom at age 27. *Intern. Med.* 31: 922-925.

Esmon CT (1987). The regulation of natural anticoagulant pathways. *Science* 235: 1348-1352.

Foley JH, Ferris L and Brummel-Ziedins KE (2012). Characteristics of fibrin formation and clot stability in individuals with congenital type IIb protein C deficiency. *Thromb. Res.* 129: e142-e146.

Formstone CJ, Hallam PJ, Tuddenham EG, Voke J, et al. (1996). Severe perinatal thrombosis in double and triple heterozygous offspring of a family segregating two independent protein S mutations and a protein C mutation. *Blood* 87: 3731-3737.

Foster DC, Yoshitake S and Davie EW (1985). The nucleotide sequence of the gene for human protein C. *Proc. Natl. Acad. Sci. U. S. A.* 82: 4673-4677.

Greengard JS, Fisher CL, Villoutreix B and Griffin JH (1994). Structural basis for type I and type II deficiencies of antithrombotic plasma protein C: patterns revealed by three-dimensional molecular modelling of mutations of the protease domain. *Proteins* 18: 367-380.

Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, et al. (1981). Deficiency of protein C in congenital thrombotic disease. *J. Clin. Invest.* 68: 1370-1373.

Hoshi S, Hijikata M, Togashi Y, Aoyagi T, et al. (2007). Protein C deficiency in a family with thromboembolism and identified gene mutations. *Intern. Med.* 46: 997-1003.

Ido M, Ohiwa M, Hayashi T, Nishioka J, et al. (1993). A compound heterozygous protein C deficiency with a single nucleotide G deletion encoding Gly-381 and an amino acid substitution of Lys for Gla-26. *Thromb. Haemost.* 70: 636-641.

Khor B and Van Cott EM (2010). Laboratory tests for protein C deficiency. *Am. J. Hematol.* 85: 440-442.

Manabe S and Matsuda M (1985). Homozygous protein C deficiency combined with heterozygous dysplasminogenemia found in a 21-year-old thrombophilic male. *Thromb. Res.* 39: 333-341.

Miletich J, Sherman L and Broze G Jr (1987). Absence of thrombosis in subjects with heterozygous protein C deficiency. *N. Engl. J. Med.* 317: 991-996.

Miyata T, Sakata T, Zheng YZ, Tsukamoto H, et al. (1996). Genetic characterization of protein C deficiency in Japanese subjects using a rapid and nonradioactive method for single-stand conformational polymorphism analysis and a model building. *Thromb. Haemost.* 76: 302-311.

Naito M, Mimuro J, Endo H, Madoiwa S, et al. (2003). Defective sorting to secretory vesicles in trans-Golgi network is partly responsible for protein C deficiency: molecular mechanisms of impaired secretion of abnormal protein C R169W, R352W, and G376D. *Circ. Res.* 92: 865-872.

Reitsma PH (1997). Protein C deficiency: from gene defects to disease. *Thromb. Haemost.* 78: 344-350.

Seligsohn U, Berger A, Abend M, Rubin L, et al. (1984). Homozygous protein C deficiency manifested by massive venous thrombosis in the newborn. *N. Engl. J. Med.* 310: 559-562.

Sugahara Y, Miura O, Yuen P and Aoki N (1992). Protein C deficiency Hong Kong 1 and 2: hereditary protein C deficiency caused by two mutant alleles, a 5-nucleotide deletion and a missense mutation. *Blood* 80: 126-133.

Sugahara Y, Miura O, Hirosawa S and Aoki N (1994). Compound heterozygous protein C deficiency caused by two mutations, Arg-178 to Gln and Cys-331 to Arg, leading to impaired secretion of mutant protein C. *Thromb. Haemost.* 72: 814-818.

Tjeldhorn L, Iversen N, Sandvig K, Bergan J, et al. (2010). Functional characterization of the protein C A267T mutation: evidence for impaired secretion due to defective intracellular transport. *BMC Cell Biol.* 11: 67.

Tridapalli E, Stella M, Capretti MG and Faldella G (2010). Neonatal arterial iliac thrombosis in type-I protein C deficiency: a case report. *Ital. J. Pediatr.* 36: 23.

Tsay W and Shen MC (2004). R147W mutation of PROC gene is common in venous thrombotic patients in Taiwanese Chinese. *Am. J. Hematol.* 76: 8-13.

Zhou RF, Cai XH, Xie S, Wang WB, et al. (2007). Molecular mechanisms of protein C deficiency caused by C64W and F139V mutations. *Zhonghua Xue Ye Xue Za Zhi* 28: 156-159.

Zhu T, Ding Q, Bai X, Wang X, et al. (2011). Normal ranges and genetic variants of antithrombin, protein C and protein S in the general Chinese population. Results of the Chinese hemostasis investigation on natural anticoagulants study I group. *Haematologica* 96: 1033-1040.