Peculiar laboratory phenotype/genotype relationship due to compound inherited protein C defects in a child with severe venous thromboembolism

Cristiana Bulato; Elena Campello; Sabrina Gavasso; Sara Maggiolo; Daniela Tormene; Paolo Simioni

Department of Medicine – DIMED, Thrombotic and Haemorrhagic Diseases Unit, Veneto Region Haemophilia and Thrombophilia Centre, University of Padua Medical School, Padua, Italy

Keywords

Protein C defects, children, thrombosis, thrombophilia, phenotype/genotype relationship

Summary

A 7-years-old child who developed unprovoked deep vein thrombosis (DVT) and pulmonary embolism (PE) was tested for inherited thrombophilia. Protein C (PC) antigen level (87 %) and PC coagulometric and amidolytic activities (12 % and 11 %, respectively) were consistent with a homozygous PC type IIA phenotype.

The patient was carrier of two heterozygous missense mutations causing p.Arg32Cys substitution associated with a type I PC defect ("null allele", from the paternal side) and p.Gly433Ser substitution responsible for a type IIA PC defect (from the maternal side). Thus, the apparently normal PC antigen level in the proband was misleading in the interpretation of phenotype/genotype relationship of this compound PC defect. The child was also carrier of heterozygous prothrombin G20210A variant.

Severe venous thromboembolism can occur in otherwise healthy children with complex inherited thrombophilia. Careful laboratory characterization of the phenotype/genotype relationship can be crucial to correctly classify PC defects and for their management with anticoagulants or replacement therapy.

Schlüsselwörter

Protein C defekten, Kinder, Thrombosis, Thrombophilie, Phänotyp-/Genotyp-Beziehung

Zusammenfassung

Ein 7-jähriges Kind wurde aufgrund einer unprovozierten tiefen Venenthrombose (TVT) und Lungenembolie (LE) auf hereditäre Thrombophilien getestet. Der Antigen-Spiegel an Protein C (PC) (87 %), sowie Aktivität von PC, welche mittels koagulometrischen und chromogenem (amidolytischem) Assay bestimmt wurde (12 % bzw. 11 %), waren mit einem homozygoten PC Typ IIA Phänotyp vereinbar.

Tatsächlich war der Patient compound heterozygot, d. h. Träger von zwei unterschiedlichen Missense-Mutationen des PC-Gens: eine p.Arg32Cys Substitution, dass mit einer type I PC Defekt („null Allel", von der väterlichen Seite) assoziiert ist, und eine p.Gly433Ser Substitution, dass für einer type II PC defekt (von der mütterlichen Seite) verantwortlich ist.

Dies führte zu einem irreführend normalen PC Antigen Spiegel bei dem Probanden im Rahmen der Interpretation der Phänotyp/Genotyp-Beziehung dieses kombinierten PC Defekts. Das Kind war zudem heterozygoter Träger der Prothrombin G20210A Mutation.

Schwere venöse Thromboembolien können bei ansonsten gesunden Kindern mit komplex vererbten Thrombophilien auftreten. Eine sorgfältige Laborcharakterisierung der Phänotyp-/Genotyp-Beziehung kann entscheidend sein für die korrekte Klassifizierung von PC-Defekten sowie für das Management mit Antikoagulantien oder Ersatztherapie.

Korrespondenzadresse
Prof. Paolo Simioni, MD, PhD
Department of Medicine – DIMED
Thrombotic and Haemorrhagic Diseases Unit
Veneto Region Haemophilia and Thrombophilia Centre
University of Padua Medical School
Via Giustiniani 2, 35128 Padua, Italy
Tel. +39 049 8212667,
mobile +39 328 8345507,
Fax +39 049 8212651
e-mail: paolo.simioni@unipd.it

Auffälligephänotyp/Genotyp-Beziehung im Labor aufgrund eines komplex vererbten Protein C Defekts bei einem Kind mit unprovozierter tiefer Venenthrombose
Hämostaseologie 2018; 38: 33–38
https://doi.org/10.5482/HAMO-17-03-0013
received: March 20, 2017
accepted in revised form: October 29, 2017

Introduction

Protein C (PC) is a natural anticoagulant whose deficiency is associated with an increased risk of venous thromboembolism (VTE). PC deficiency can be transmitted as an autosomal dominant or autosomal recessive trait. The prevalence of mild (heterozygous forms) PC deficiency in the healthy population ranges from 1/200 to 1/500 (1). Heterozygous carriers are often asymptomatic, but may experience recurrent thrombotic events in the presence of additional inherited prothrombotic risk factors, such as factor V (FV) Leiden or

prothrombin (PT) G20210A mutations (2). In contrast, severe PC deficiency (homozygous and double heterozygous forms) is very rare (from 1/40000 to 1/250000) (3) and manifests in the neonatal period by purpura fulminans, disseminated intravascular coagulation and massive venous thrombosis (4) with rare exceptions.

Based on the functional and immunological assays, PC deficiency can be classified in three types. Type I deficiency, the more common form, is quantitative ("true" defect) and type II deficiency is qualitative (dysfunctional molecules). Type I deficiency is characterized by a concordant reduction in PC antigen and PC coagulometric and amidolytic activities. In contrast, type II deficiency exhibits normal PC antigen but the function of the molecule is impaired. Type II is further classified into two subtypes: type IIA, in which both coagulometric and amidolytic activities are reduced (when the lesion affects the catalytic site) (5), and type IIB, in which only the coagulometric activity is reduced (when the lesion affects the gamma-carboxyglutamic acid-rich (Gla) domain or impairs the interaction of PC with its physiological substrates factors V and VIII) (6–8). Type III deficiency results from the combination in the heterozygous form of a type I defect with a type II defect, which leads to a reduced synthesis of a dysfunctional PC molecule. Type III deficiency shows a discrepancy between PC antigen and PC activities with a greater decrease in the level of both activities compared to the antigen level (9) (▶Tab. 1).

Here, we present a peculiar compound PC defect in combination with heterozygous PT G20210A mutation found in an Italian child with severe thrombotic manifestations.

Case report and results

A 7-year-old boy was admitted to our Pediatric Emergency Department in 2008 for a painful swelling of his leg appeared about 7 days after the onset of varicella.

Compression ultrasonography of the lower limbs revealed complete thrombosis of the right popliteal, superficial femoral, common femoral and iliac veins, with involvement of the right small and great saphenous veins, and almost complete thrombosis of the left small and great saphenous veins up to the cross with the left superficial femoral vein. Perfusion lung scan did not show any significant perfusion defect.

His family history was positive for thromboembolic events, since his father had suffered two episodes of deep vein thrombosis (DVT) in the right leg complicated by pulmonary embolism (PE).

Routine coagulation tests, performed in the patient on admission, yielded normal values except for the PC. PC antigen was 87% (normal range, 80–120%) whereas PC activity, measured by both coagulometric and amidolytic assays, were markedly reduced to 12% and 11%, respectively (normal range for both activities, 80–120%). The child was also heterozygous for PT G20210A mutation. These preliminary findings suggested that the patient suffered from severe PC deficiency.

Therapeutic dose of subcutaneous low molecular weight heparin (LMWH) was started and after a few days the warfarin therapy was initiated. Compression ultrasonography was performed after a few days and, despite a partial recanalization of the veins of the right leg, an extension of thrombosis to the deep veins of the left leg was observed.

Because both PC coagulometric and amidolytic activities had fallen to undetectable levels, PC concentrates (Ceprotin®, Baxter, Vienna, Austria) were administered at the dose of 50 IU/Kg in association with intravenous heparin. Six days later, warfarin was added to the heparin treatment and, after an additional overlap of 1 week, international normalized ratio (INR) values were in therapeutic range (INR 2.0 to 3.0) and both heparin and PC concentrates were discontinued.

After 2 weeks of hospitalization, the child presented with sudden right-sided chest pain, fever, cough and difficulty breathing. Because of the clinical suspect of PE, lung perfusion scinti-scan was performed, which showed no perfusion of the right lung in the presence of a normal chest X-ray (mismatch). Warfarin was adjusted to achieve and maintain a therapeutic INR range of 2.5–3.5 and new infusions of PC concentrates (2000 IU) were scheduled every 3 days. At this dosage, a trough level of PC activity was kept around 20% in this child. The patient's symptoms improved in the subsequent 2 weeks and he was discharged. No recurrences were observed in the following 6 months and PC concentrates were then discontinued without further VTE.

All available family members (▶Fig. 1) underwent coagulation screening. The levels of PC antigen and activities found in the proband (III.1) and his relatives are summarized in ▶Tab. 2.

The proband's father (II.1) (who was under warfarin therapy) and the proband's grandfather (I.1) showed a concordant decrease in PC antigen and activities reflecting a heterozygous type I PC defect. The proband's father was also found to be heterozygous for PT G20210A mutation.

Tab. 1 Classification of inherited protein C (PC) defects

Classification	PC antigen	PC amidolytic activity	PC coagulometric activity
Type I (quantitative or true defects of PC)	Reduced	Reduced	Reduced
Type IIA (qualitative or dysfunctional defects of PC)	Normal	Reduced	Reduced
Type IIB (qualitative or dysfunctional defects of PC)	Normal	Normal	Reduced
Type III or Hypo-dys (quantitative and qualitative defects of PC)	Reduced	Reduced (lower than the antigen level)	Reduced (lower than the antigen level)

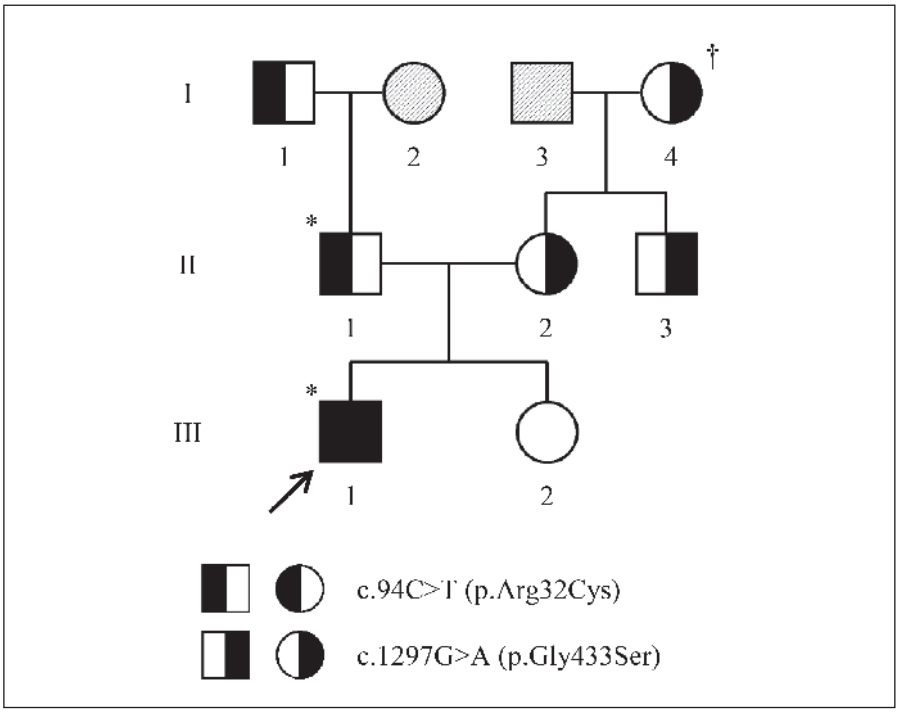

Fig. 1 Family pedigree showing protein C genotypes. The proband was indicated by an arrow. Half-filled symbols indicate individuals heterozygous for c.94C>T (p.Arg32>Cys) or c.1297G>A (p.Gly433Ser) mutation. Solid symbols indicate individuals heterozygous for both mutations. Open symbols indicate normal individuals. Symbols with diagonal lines indicate unexplored subjects. Circles represent females and squares represent males. *individuals heterozygous for prothrombin G20210A mutation; †individuals heterozygous for factor V Leiden mutation. III-1 (proband) and II-1 (proband's father) are the only symptomatic family members for venous thromboembolism.

The proband's mother (II.2) and the maternal uncle (II.3) showed PC antigen levels slightly above the upper limit of the normal range (134 % and 139 %, respectively), whereas both PC coagulometric and amidolytic activities were reduced to approximately 40–50 % of normal range.

The maternal grandmother (I.4), who was also heterozygous for FV Leiden mutation, exhibited a PC antigen slightly below the normal range (75 %), whereas PC coagulometric and amidolytic activities were decreased to 24 % and 22 %, respectively. Coagulation screening of the maternal grandmother revealed multiple defects of synthesis possibly related to liver failure. Except for the patient's father, all other family members were asymptomatic for VTE.

Direct sequencing of the PC gene, performed as previously described (10), revealed that the proband was compound heterozygous for two known mutations: c.94C>T (p.Arg32Cys) and c.1297G>A (p.Gly433Ser) localized on exon 3 and 9, respectively. The heterozygous p.Arg32Cys substitution was also detected in the proband's father and paternal grandfather, whereas the proband's mother, maternal uncle and maternal grandmother were heterozygous for p.Gly433Ser substitution. The proband's sister did not carry any of these mutations. Sequence analysis of the promoter showed three well-documented polymorphisms: rs1799808, rs1799809 and rs1799810 (also termed –1654C/T, –1641G/A and –1476A/T, respectively, according to the numbering of Foster et al. (11)). Haplotypes found in the individuals examined are presented in ▶Tab. 2.

Discussion

Protein C plays a critical role in physiological anticoagulation and congenital PC deficiency is a well-known risk factor for VTE in adults (12). Heterozygous subjects have PC levels half of normal and may develop thromboembolic events in early adulthood or remain asymptomatic throughout life (12). Homozygous or compound heterozygous individuals have very low or undetectable PC levels and may present with purpura fulminans and disseminated intravascular coagulation in the neonatal period (13, 14).

In this study, a severe PC defect was detected in a child with DVT in both lower limbs complicated by PE. In order to characterize the defect, family study was performed, which revealed two different PC gene lesions resulting in peculiar phenotypes. The proband's father and other family members on this side showed a classical type I heterozygous PC deficiency due to p.Arg32Cys mutation (15–17). The proband's mother and other family members on this side showed a type IIA heterozygous PC deficiency due to p.Gly433Ser mutation (16). Interestingly enough, the carriers of this mutation in our family presented with both PC coagulometric and amidolytic activities reduced to about 40–50 % of normal, whereas antigen levels were higher than normal upper limit (134 % and 139 %, respectively). One explanation could be an overexpression of PC related to the mutation in itself or to genetic variations present in the PC gene promoter. DNA sequencing of the promoter region in carriers of p.Gly433Ser mutation revealed three common polymorphisms, known to influence the plasma antigen levels of PC (rs1799808, rs1799809 and rs1799810). However, these polymorphisms have been associated with low PC antigen levels (18, 19). Notably, different genome-wide association studies (GWAS) support the idea that polymorphisms in other genes may affect circulating levels of PC (20, 21). Another possible explanation of the increased antigen levels may be related to the type of antibodies used in the ELISA test (22) and their possible interaction with PC in plasma of carriers of p.Gly433Ser mutation. In other words, the mutation could result in an increased affinity for the antibodies causing an apparent higher PC antigen level in the ELISA test. Very interestingly, higher PC antigen levels were previously reported by Reitsma et al. in some probands affected by the same heterozygous mutation (16). Needless to say,

Tab. 2 Protein C phenotype and genotype of the proband and family members

Individual	PC antigen, %[1]	PC amidolytic activity, %[1]	PC coagulometric activity, %[1]	c.94C>T p.Arg32Cys	c.1297G>A p.Gly433Ser	Promoter haplotypes			Symptoms
						rs1799808	rs1799809	rs1799810	
III.1, proband	87	11	12	Heterozygous	Heterozygous	CC	GG	TT	DVT + PE
II.1, father[2]	30	32	12	Heterozygous	Normal	CC	GA	TT	DVT
II.2, mother	134	49	45	Normal	Heterozygous	CT	GA	AT	Asymptomatic
III.2, sister	98	94	82	Normal	Normal	CT	AA	AA	Asymptomatic
I.1, paternal grandfather	49	52	46	Heterozygous	Normal	CT	GA	AT	Asymptomatic
I.4, maternal grandmother	75	22	24	Normal	Heterozygous	CT	GA	AT	Asymptomatic
II.3, maternal uncle	139	47	43	Normal	Heterozygous	CC	GG	TT	Asymptomatic

Nucleotide variations and amino acid changes were described according to the Human Genome Variation Society. [1]Normal range for protein C antigen and activities, 80–120 %. [2]On oral anticoagulant therapy at the time of blood sampling. PC, protein C; DVT, deep vein thrombosis; PE, pulmonary embolism.

different anti-PC antibodies (monoclonal/polyclonal) in the ELISA assay could result in different levels of PC antigen.

What could it be the result of the combination of these two PC gene lesions in the same patient in terms of laboratory phenotype? Sequencing results in the proband showed the presence of one null allele (p.Arg32Cys mutation) and a second allele (p.Gly433Ser mutation) responsible for the synthesis of a dysfunctional PC molecule. Contrary to what expected, however, PC antigen was about 87 % and PC activities around 10 %. These findings are related to the effect of p.Gly433Ser mutation on laboratory tests for PC and no contribution to PC antigen levels is given by the null allele (p.Arg32Cys mutation) causing the lack of normal PC in the proband's plasma. The combination of these two PC defects in the proband accounts for a so called "pseudo-homozygous p.Gly433Ser mutation" with a laboratory phenotype suggestive of a severe (homozygous) type IIA PC defect. The proband was also a heterozygous carrier of PT G20210A mutation. The presence of this variant, in addition to the two lesions identified in the PC gene, could have contributed to the onset of thrombosis. Other risk factors, including recent infections (varicella, even though no autoantibodies towards PC or protein S have been detected (23)) and abnormal response to the initial anticoagulant treatment (warfarin can further reduce PC levels during its administration and cause further unbalance towards hypercoagulability), could have been responsible for the initial thrombotic events extension in this child. Thrombotic manifestations occurring in otherwise healthy children require combinations of several concomitant risk factors including severe inherited thrombophilia (24, 25) such as in our proband. Healthy children who are carriers of thrombophilia, however, may remain asymptomatic also in the presence of common inherited thrombophilic conditions (26).

Conclusion

The identification of the specific mutations underlying severe thrombotic manifestations, particularly in children, may help making an accurate diagnosis and providing patients with appropriate therapies. In many cases, both laboratory genotyping and phenotyping are required for a correct classification of PC defects together with an extensive investigation of family members, which includes both laboratory testing and collection of accurate clinical information.

References

1. Tait RC, Walker ID, Reitsma PH, et al. Prevalence of protein C deficiency in the healthy population. Thromb Haemost 1995; 73: 87–93.
2. Tirado I, Mateo J, Soria JM, et al. Contribution of prothrombin 20210A allele and factor V Leiden mutation to thrombosis risk in thrombophilic families with other hemostatic deficiencies. Haematologica 2001; 86: 1200–1208.
3. Yang LH, Zheng FX, Chen Y, et al. The significance of F139V mutation on thrombotic events in compound heterozygous and homozygous protein C deficiency. Blood Coagul Fibrinolysis 2014; 25: 824–830.
4. Goldenberg NA, Manco-Johnson MJ. Protein C deficiency. Haemophilia 2008; 14: 1214–1221.
5. Girolami A, Simioni P, Lazzaro AR, Girolami B, Prandoni P. A family with an abnormal protein C and a thrombotic tendency. Haematologia (Budap) 1993; 25: 25–33.
6. Girolami A, Simioni P, Girolami B, et al. A novel dysfunctional protein C (protein C Padua 2) associated with a thrombotic tendency: substitution of Cys for Arg-1 results in a strongly reduced affinity for binding of Ca++. Br J Haematol 1993; 85: 521–527.
7. Wojcik EG, Simioni P, d Berg M, Girolami A, Bertina RM. Mutations which introduce free cysteine residues in the Gla-domain of vitamin K dependent proteins result in the formation of complexes with alpha 1-microglobulin. Thromb Haemost 1996; 75: 70–75.
8. Simioni P, Kalafatis M, Tormene D, et al. Abnormal propeptide processing resulting in the presence of two abnormal species of protein C in plasma: characterization of the dysfunctional protein C Padua3 (protein C(R-1L/propeptide)). Thromb Haemost 2001; 86: 1017–1022.

9. Simioni P, Kalafatis M, Millar DS, et al. Compound heterozygous protein C deficiency resulting in the presence of only the beta-form of protein C in plasma. Blood 1996; 88: 2101–2108.
10. Bulato C, Radu CM, Campello E, et al. New prothrombin mutation (Arg596Trp, prothrombin Padua 2) associated with venous thromboembolism. Arterioscler Thromb Vasc Biol 2016; 36: 1022–1029.
11. Foster DC, Yoshitake S, Davie EW. The nucleotide sequence of the gene for human protein C. Proc Natl Acad Sci U S A 1985; 82: 4673–4677.
12. Simioni P, Sanson BJ, Prandoni P, et al. Incidence of venous thromboembolism in families with inherited thrombophilia. Thromb Haemost 1999; 81: 198–202.
13. Seligsohn U, Berger A, Abend M, et al. Homozygous protein C deficiency manifested by massive venous thrombosis in the newborn. N Engl J Med 1984; 310: 559–562.
14. Marciniak E, Wilson HD, Marlar RA. Neonatal purpura fulminans: a genetic disorder related to the absence of protein C in blood. Blood 1985; 65: 15–20.
15. Cafolla A, D'Andrea G, Baldacci E, Margaglione M, Mazzucconi MG, Foa R. Hereditary protein C deficiency and thrombosis risk: genotype and phenotype relation in a large Italian family. Eur J Haematol 2012; 88: 336–339.
16. Reitsma PH, Bernardi F, Doig RG, et al. Protein C deficiency: a database of mutations, 1995 update. On behalf of the Subcommittee on Plasma Coagulation Inhibitors of the Scientific and Standardization Committee of the ISTH. Thromb Haemost 1995; 73: 876–889.
17. Ireland HA, Boisclair MD, Taylor J, et al. Two novel (R(-11)C; T394D) and two repeat missense mutations in the protein C gene associated with venous thrombosis in six kindreds. Hum Mutat 1996; 7: 176–179.
18. Spek CA, Koster T, Rosendaal FR, Bertina RM, Reitsma PH. Genotypic variation in the promoter region of the protein C gene is associated with plasma protein C levels and thrombotic risk. Arterioscler Thromb Vasc Biol 1995; 15: 214–218.
19. Aiach M, Nicaud V, Alhenc-Gelas M, et al. Complex association of protein C gene promoter polymorphism with circulating protein C levels and thrombotic risk. Arterioscler Thromb Vasc Biol 1999; 19: 1573–1576.
20. Tang W, Basu S, Kong X, et al. Genome-wide association study identifies novel loci for plasma levels of protein C: the ARIC study. Blood 2010; 116: 5032–5036.
21. Pintao MC, Roshani S, de Visser MC, et al. High levels of protein C are determined by PROCR haplotype 3. J Thromb Haemost 2011; 9: 969–976.
22. Campello E, Spiezia L, Radu CM, et al. Circulating microparticles and the risk of thrombosis in inherited deficiencies of antithrombin, protein C and protein S. Thromb Haemost 2016; 115: 81–88.
23. Regnault V, Boehlen F, Ozsahin H, et al. Anti-protein S antibodies following a varicella infection: detection, characterization and influence on thrombin generation. J Thromb Haemost 2005; 3: 1243–1249.
24. Tormene D, Gavasso S, Rossetto V, Simioni P. Thrombosis and thrombophilia in children: a systematic review. Semin Thromb Hemost 2006; 32: 724–728.
25. Young G, Albisetti M, Bonduel M, et al. Impact of inherited thrombophilia on venous thromboembolism in children: a systematic review and meta-analysis of observational studies. Circulation 2008; 118: 1373–1382.
26. Tormene D, Simioni P, Prandoni P, et al. The incidence of venous thromboembolism in thrombophilic children: a prospective cohort study. Blood 2002; 100: 2403–2405.