RESEARCH LETTER

Five new mutations in the *PROS1* gene associated with protein S deficiency in Polish patients screened for thrombophilia: efficacy of direct oral anticoagulant treatment

Ewa Wypasek[1,2*], Daniel P. Potaczek[3*], Adrianna Klajmon[1], Angel Zúñiga[4], Anetta Undas[1,5]

1 Laboratory of Molecular Biology, John Paul II Hospital, Kraków, Poland
2 Faculty of Medicine and Health Sciences, Andrzej Frycz Modrzewski Krakow University, Kraków, Poland
3 Translational Inflammation Research Division & Core Facility for Single Cell Multiomics, Medical Faculty, Biochemical Pharmacological Center (BPC), Philipps University of Marburg, Marburg, Germany
4 Genetics Unit, Hospital Universitario y Politécnico La Fe, Valencia, Spain
5 Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland

Correspondence to:
Ewa Wypasek, PhD, Laboratory of Molecular Biology, John Paul II Hospital, ul. Prądnicka 80, 31-202 Kraków, Poland, phone: +48 12 614 31 45, email: e.wypasek@szpitaljp2.krakow.pl
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* EW and DPP contributed equally to this work.

Introduction Protein S is a vitamin K–dependent glycoprotein that serves as a cofactor of activated protein C in the proteolytic inactivation of activated factor V and factor VIII by their cleavage at the specific arginine residues.[1]

In most cases, congenital protein S deficiency is inherited in an autosomal dominant pattern with variable penetrance and frequency of 0.5% in the general European population and of 2% to 12% in patients with thrombosis.[2] Individuals with heterozygous mutations causing protein S deficiency have a 10-fold higher risk of venous thromboembolism (VTE) compared with their healthy relatives.[2] Evidence linking this thrombophilic factor with myocardial infarction or ischemic stroke is limited.[3,4] Based on the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), 450 *PROS1* mutations were described as causes of protein S deficiency, with a predominance of missense / nonsense mutations (56%).

Previously, we were the first to describe the Polish cases of genetically confirmed protein S deficiency.[5-7] Here, we report a series of 12 protein S–deficient Polish patients with their clinical and genetic characterization along with long-term follow-up data, including 5 new *PROS1* mutations associated with this thrombophilia.

Patients and methods We identified 12 unrelated patients (probands) with a personal history of thromboembolic events (n = 11) and / or positive family history (n = 6) with a suspicion of protein S deficiency in the Center for Coagulation Disorders at the John Paul II Hospital, Kraków, Poland, between January 2018 and December 2020. The family history of VTE in the first and / or second degree relatives of the probands was collected. Family history was regarded positive if VTE was diagnosed in at least 1 relative. The diagnoses of VTE and ischemic stroke were established as previously described.[5]

The patients were followed until April 2021. Data on thromboembolic events or bleeding (based on the International Society on Thrombosis and Hemostasis criteria) were collected at regular clinic visits and on telephone contact every 6 to 12 months. The retrospective analysis was part of clinical diagnostic evaluation, and thus, the approval of a bioethical committee was not required.

Laboratory tests The tests were performed at least 3 months after the thromboembolic event and after temporary withdrawal of a vitamin K antagonist (for at least 10 days) or direct oral anticoagulant (for at least 24 hours). The probands were screened for thrombophilia as described previously.[4] Free protein S (FPS) levels were measured using an immunoturbidimetric assay (INNOVANCE Free protein S Ag, Siemens Healthcare Diagnostic, Marburg, Germany; reference range, 60%–114% for women and 67%–139% for men). Total protein S levels were assessed using the Asserachrom kit (Diagnostica Stago, Asnieres, France; reference range, 75%–140%). Protein S deficiency was classified as previously described.[5]

Genetic analysis Genomic DNA was isolated from peripheral blood using an extraction kit

following standard procedure (QIAsymphony, Qiagen, Hilden, Germany or gene MATRIX Quick Blood DNA Purification Kit, Eurex, Gdańsk, Poland) and stored at –80 °C until analysis. A custom SureSelect panel was designed for the *PROS1* gene using SureDesign Custom Design Tool (both Agilent Technologies, Santa Clara, California, United States). An adaptor-tagged DNA library was purified, amplified, and enriched using the SureSelect XT capture library (Agilent Technologies). Sequencing of the libraries was performed on a NextSeq 550 instrument (Illumina, San Diego, California, United States). All variants detected were confirmed by Sanger sequencing. Primers were designed in order to avoid pseudogene amplification. Mutations were given coordinates according to the Human Genome Organisation recommendations for mutation nomenclature (http://www.hgvs.org). The *PROS1* gene cDNA (NM_001314077.2) and protein (NP_001301006.1) sequences were retrieved from the National Center for Biotechnology Information Reference Sequence database.

In silico analysis Each novel sequence variation was subjected to in silico analyses with: Polymorphism Phenotyping, version 2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2) and VarSome (https://varsome.com) for missense mutations, NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2) for exon-intron boundary variants, or miRBase (http://www.mirbase.org) and RNA Analyzer (https://rnaanalyzer.bioapps.biozentrum.uni-wuerzburg.de) for the 3' untranslated region (3' UTR) mutation.

Results As shown in TABLE 1, there were 12 protein S–deficient probands (mean [SD] age, 35.8 [7.8] years; 50% men) without any other thrombophilias. The first VTE event was diagnosed at the mean (SD) age of 30.4 (8.1) years in 11 symptomatic patients with a history of thromboembolism. Positive family history was found in 6 patients (50%).

The mean (SD) FPS levels were 43.5% (16.9%) and the mean (SD) total protein S antigen was 64.8% (14.4%). Type I protein S deficiency was detected in 11 patients and type II in 1 patient.

The most common clinical manifestation of protein S deficiency was isolated deep vein thrombosis (n = 5). Four VTE events were provoked by long journey or surgery, and 2 events by oral contraception.

Arterial thromboembolic events, that is, ischemic stroke, occurred in 2 patients without prior VTE. With regard to anticoagulant treatment, 6 patients (50%) were on rivaroxaban (20 mg/d), 1 on apixaban (2.5 mg twice daily), 1 on dabigatran (150 mg twice daily), 2 on acetylsalicylic acid, and 1 on acenocoumarol.

During follow-up (median [interquartile range], 21 [3–42] months), 1 thromboembolic event, that is, left hemisphere infarction, occurred 14 months after the first stroke (patient 8). The patient was on acetylsalicylic acid 150 mg daily and stopped smoking after the first incident. The patient was switched to dabigatran 150 mg twice daily with no thromboembolic events observed so far. No major or clinically relevant nonmajor bleedings were reported.

***PROS1* mutations** Nine patients (75%) carried missense *PROS1* mutations, 2 patients (16.7%) had splice-site variants, and 1 individual (8.3%) a 3'-UTR mutation. Interestingly, the p.Thr110Met variant was identified in 4 patients. To the best of our knowledge, we report for the first time 5 mutations (42%) in patients 1 to 5 (TABLE 1).

Discussion We described 12 patients with protein S deficiency, 5 of whom were identified as carriers of newly detected *PROS1* mutations. In silico analysis indicated that 4 of the new mutations are likely to be detrimental, which corresponds to thrombotic manifestations of protein S deficiency.

The p.Arg362Trp mutation (in patient 1) was predicted to be possibly (PolyPhen-2 score of 0.801) or probably (PolyPhen-2 score of 1.000) damaging, depending on the analyzed isoform (NP_001301006.1 or NP_000304.2, respectively). Those results were even more striking for the p.Leu478Pro mutation (patient 2), which was predicted to be probably damaging with the PolyPhen-2 score of 1.000 for both protein isoforms. In addition, 20 and 19 out of 21 scores calculated by VarSome indicated pathogenicity of the p.Arg362Trp and p.Leu478Pro mutations, respectively. Thus, the functional effects of the p.Arg362Trp and p.Leu478Pro missense mutations are highly likely and in line with our laboratory and clinical observations.

In turn, genetic variants c.76+2_76+3del (patient 3) and c.1966+1delG (patient 4) prevent normal splicing by destroying respective canonical donor splice sites. This can lead to the formation of transcripts with included introns, partial deletions, or entirely skipped exons, yielding nonfunctional, for example, truncated, proteins.[8] Interestingly, c.76+2_76+3del damages the donor site directly preceding an additional exon, the presence or absence of which differentiates between the NM_001314077.2 and NM_000313.4 transcript variants, which might also be involved in impaired protein S synthesis. In any case, c.76+2_76+3del and c.1966+1delG seem to be functionally responsible for the clinical and biochemical effects we observed.

The last new mutation, c.*783C>T 3'UTR (patient 5), is of even more interest. First, our analyses demonstrated that the variant allele removes the binding sites for 2 human microRNAs, hsa-miR-6739-5p and hsa-miR-7153-5p. Second, in silico analysis strongly suggested that the presence of the mutant allele is associated with the formation of the new polyadenylation signal, located about 300 nucleotides upstream of the canonical polyadenylation signal, thus potentially

TABLE 1 Characteristics of patients with protein S deficiency

Patient no.	Sex/age	Free protein S, %	Total protein S, %	Type of protein S deficiency	Type of mutation	New/reported	Clinical manifestation	Age at the first VTE event	VTE events, n	Unprovoked/provoked (for the first event)	Family history of VTE	Duration, mo	Thromboembolic events	Antithrombotic treatment
1	M/27	48; 48	70	I	c.1084C>T p.Arg362Trp	New	PE	18	1	0/1 (surgery)	1	42	0	Apixaban, 2×2.5 mg/d
2	M/23	33; 25	53	I	c.1433T>C p.Leu478Pro	New	DVT	21	1	1/0	0	27	0	Rivaroxaban, 20 mg/d
3	M/36	10; 11[a]	ND	I	c.76+2_76+3del	New	DVT	28	2	0/1 (long journey)	1	12	0	Acenocoumarol, 3–4 mg/d
4	M/28	24; 34	51	I	c.1966+1delG	New	DVT + PE	21	1	1/0	0	17	0	Rivaroxaban, 20 mg/d
5	F/45	29; 53	51	I	c.*783C>T 3'UTR	New	Asymptomatic	–	–	–	1	–	–	–
6	F/35	26; 24	67	I	c.1639C>T p.Arg547Cys	Reported	DVT + PE	32	3	1/0	0	4	0	Rivaroxaban, 20 mg/d
7	M/45	59; 54	68	I	c.296A>C p.Glu99Ala	Reported	DVT	43	2	1/0	1	3	0	Rivaroxaban, 20 mg/d
8	F/43	58; 47	63	I	c.1597T>C p.Ser533Pro protein S Heerlen	Reported	2 × ischemic stroke	40	1	1/0	0	24	1 (ischemic stroke)	Dabigatran, 2×150 mg/d
9	M/45	54; 52	66	I	c.329C>T p.Thr110Met	Reported	DVT	32	4	1/0	0	40	0	Rivaroxaban, 20 mg/d
10	F/34	57; 55	58	II	c.329C>T p.Thr110Met	Reported	Ischemic stroke	33	1	1/0	1	28	0	Acetylsalicylic acid, 150 mg/d
11	F/40	66; 69	78	II	c.329C>T p.Thr110Met	Reported	DVT	38	1	0/1 (contraception)	1	7	0	Acetylsalicylic acid, 75 mg/d
12	F/29	52; 51	52	I	c.329C>T p.Thr110Met	Reported	Axillary vein thrombosis + PE	28	1	0/1 (contraception)	0	21	0	Rivaroxaban, 20 mg/d

a Determined on acenocoumarol

Abbreviations: DVT, deep vein thrombosis; F, female; M, male; ND, not determined; PE, pulmonary embolism; VTE, venous thromboembolism

leading to the formation of the transcript with much shorter 3' UTR. Usually, messenger RNA with shorter 3' UTR possess much less microRNA binding sites, which renders them more stable and thus associated with much higher gene expression.[9] Hence, paradoxically, the c.*783C>T 3'-UTR mutation would lead to higher *PROS1* messenger RNA stability and expression, at least through the 2 mechanisms mentioned above.

Four mutations (p.Arg547Cys, p.Glu99Ala, protein S Heerlen, and p.Thr110Met; patients 6–12) had been previously described in European populations and found to be associated with protein S deficiency.[10] Interestingly, the p.Thr110Met variant was detected first time in the Polish population in 4 unrelated protein S–deficient patients (patients 9–12).

From a practical point of view, all the current mutations except one (patient 11) were found in individuals with FPS levels below 55%, which is in line with a study in the German population.[11]

During follow-up, we observed a single recurrent thromboembolic event, that is, ischemic stroke (patient 8). It has been shown that those with protein S deficiency are at a higher risk of arterial thromboembolism before 55 years of age compared with nondeficient family members.[3] Our case might support growing evidence for the role of protein S deficiency in stroke pathophysiology and a value of anticoagulation in therapy of such patients, not antiplatelet agents. Moreover, our study supports the efficacy and safety of direct oral anticoagulants for venous and arterial thrombosis in the setting of inherited thrombophilias, including protein S deficiency,[12] and highlights an increasingly common use of those drugs in everyday practice also in thrombophilic individuals.[13] No bleeding, including heavy menstrual bleeding, was reported.[14,15] Importantly, our results indicate that patients after cryptogenic stroke should be considered candidates for inherited thrombophilia screening to exclude protein S deficiency with the subsequent genotyping if protein S levels are less than 55%.

The genetic etiology of thrombophilia in Poland still remains strongly underdiagnosed. We encourage an in-depth diagnostic evaluation of patients who are suspected of inherited thrombophilia, also to expand our knowledge about the genetics of thrombosis.

ARTICLE INFORMATION

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