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Letter to the Editors-in-Chief

PROS1 IVS10 + 5G > A mutation causes hereditary protein S deficiency in a Chinese patient with pulmonary embolism and venous thromboembolism

Protein S (PS, OMIM #176880) is a vitamin K-dependent plasma glycoprotein that acts as a non-enzymatic cofactor for activated protein C (PC) involved in the degradation of coagulation factors V and VIII, thereby inhibiting blood clotting [1]. PS deficiency (PSD, OMIM#612336) is an autosomal dominant (AD) haploinsufficiency disorder with incomplete penetrance and variable clinical expressivity, recognized as an independent risk factor for venous thromboembolism (VTE) and found in 1.5% to 7% of selected groups of thrombophilic patients [2].

Hereditary PSD is caused by mutation of the *PROS1* gene located at 3q11.2, comprising 15 exons and spanning over 80 kb of genomic DNA. Up to date, a total of 417 *PROS1* mutations were included in o Human Gene Mutation Database (HGMD). Missense/nonsense mutation accounts for about 50% of all mutations. The non-classical *PROS1* IVS10 + 5G > A splice site mutation has been reported in several Caucasian patients previously, however, its effect on *PROS1* mRNA splicing was still unknown [3,4].

A 46 years old male Chinese patient was admitted to our hospital for edema of lower extremity. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and DD-Dimer were tested. Blood routine tests showed that the patient's hemoglobin was 15.1 g/dL, platelet count was 135 × 10^9/L, and the hematology-coagulation tests showed increased DD-Dimer and decreased PS activity determined with a Staclot Protein S kit (Diagnostica Stago). (Table 1). Results of lupus anticoagulants Silica Clotting Time (SCT) and Russell viper venom time (RVVT) were both normal. Pulmonary artery and its branch embolism in anterior inferior basal segment of right lower lobe was observed by Computed tomography (CT) scanning (Fig. 1A). Thrombosis of the right femoral vein, superficial femoral vein and its branches was detected by color Doppler ultrasound respectively (Fig. 1B). Therefore, a diagnosis of pulmonary embolism (PE) and venous thromboembolism (VTE) partially due to protein S deficiency (PSD) was established. The patient received minimally invasive thrombolytic surgery, and the VTE was ameliorated.

To explore the genetic cause of PSD in this patient, all 15 exons and the boundary 5-bp intronic sequences of the *PROS1* gene were detected by Sanger sequencing. A heterozygous non-classical *PROS1* IVS10 + 5G > A splice site mutation (NM_000313.3: c.1155 + 5G > A) was identified in the patient (Fig. 1C). This heterozygous mutation was also found in his 23 years old daughter who also showed decreased PS activity (16%), whereas this mutation was absent from his 18 years old son with normal PS activity (99%).

To further investigate the effect of this non-classical *PROS1* IVS10 + 5G > A splice site mutation on *PROS1* mRNA splicing. RNAs isolated from peripheral blood mononuclear cells (PBMCs) of the patient and a healthy control subject was reverse transcribed into cDNA. Primers covering exon 8 to exon 12 of PROS1 mRNA were designed (Forward: AAAGGATTCAAACTTGCCCA, Reverse: TCCACAGTAACCAGGCAATG). A deletion of the last 32 nucleotides of exon 10 (NM_000313.3: c.1124_1155delGTGATGTTATTAATAATGGTCTATGGAATATG) was found in the patient but not the healthy control subject (Fig. 1D), although the mutant sequence had much lower signals shown in the electropherogram compared with the wild type sequence. The detection of cDNA was not performed in his daughter who also carried *PROS1* IVS10 + 5G > A mutation. The deletion of exon 10 resulted in a frame-shift deletion and premature termination of PS (p.Asp376ValfsTer8). In silico prediction with Human Splicing Finder 3.0 also suggested that *PROS1* IVS10 + 5G > A mutation predicted a new cryptic donor site, which may generate a frame-shift deletion (Fig. 1E). The score for splicing using the WT donor sequence of exon 10 (85.5%) is disrupted by the intronic mutation (to 73.3%), which redirect the spliceosome to a cryptic exon donor site with higher score (82.8%). This prediction was validated by RNA analysis of PBMC.

PS precursor contains 676 amino acids, and mature PS contains 635 amino acid residues consisting of a γ-carboxyglutamic acid domain (Gla), a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains (EGF1-4), and a large domain homologous to sex hormone binding globulins (SHBG-like domain). Approximately 60% of circulating PS is non-covalently bound to C4b-binding protein (C4BP) and remaining 40% serves as a free anticoagulant protein. PSD is classified as type I (reduced total and free antigen plus reduced PS activity), type II (normal total and free antigen plus low anticoagulant activity), and type III (normal total antigen plus low free antigen and reduced PS activity). Types I and II deficiencies account for 95% all PSD cases [5].

In thrombophilic families, PSD was clearly associated with venous thrombosis. Sequencing exons and splice site of *PROS1* has successfully identified mutations in approximately 50% of families with PSD. Gross deletions/insertions of *PROS1* were found in about 30% of the point

Table 1
Results of coagulation tests.

PT	13.2	11.5–14.5 s
PTA	98.0	45–125%
INR	1.01	0.80–1.20
FIB	3.20	2.00–4.00 g/L
APTT	37.3	29.0–42.0 s
TT	15.8	14.0–19.0 s
DD-Dimer	2.54 (↑)	< 0.5 μg/mL
AT	96	80–120%
PC	97	70–142%
PS	19 (↓)	77–143%

PT: prothrombin time, PTA: prothrombin activity, INR: international normalized ratio, FIB: fibrinogen, APTT: activated partial thromboplastin time, TT: thrombin time, AT: anti-thrombin III, PC: protein C, PS: protein S.

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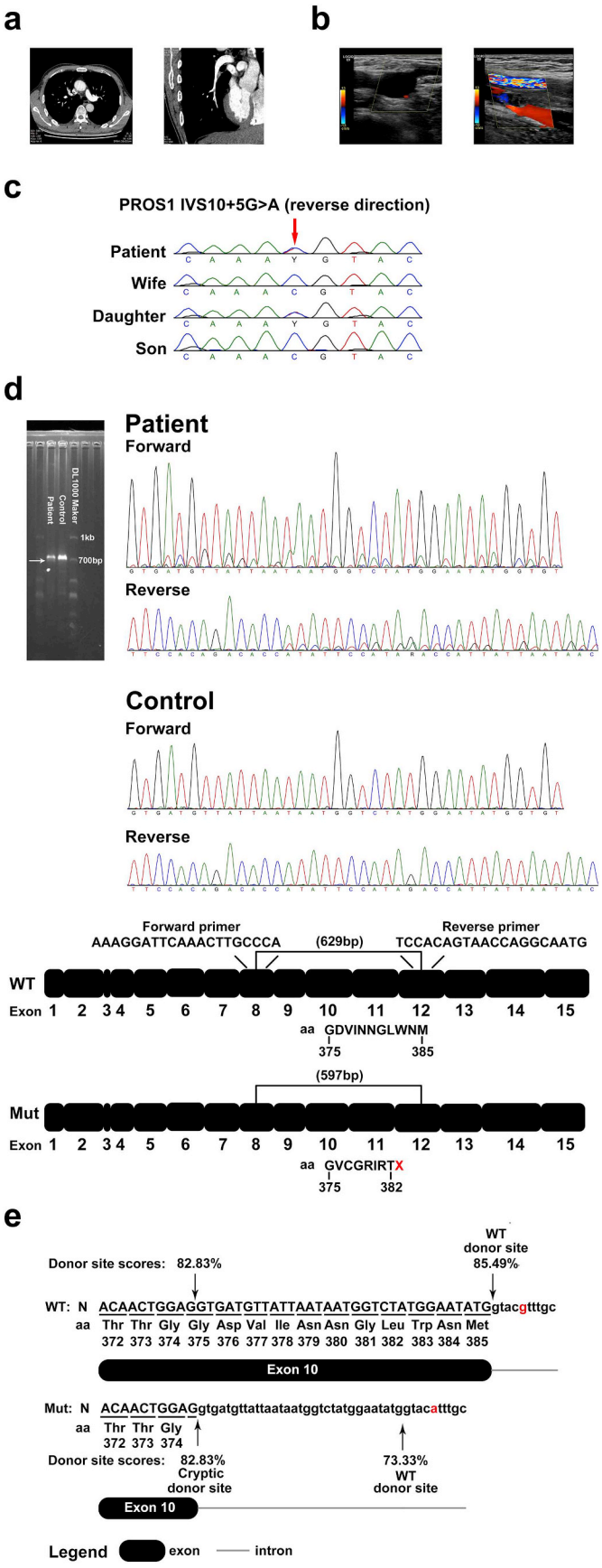
a
b
c
PROS1 IVS10+5G>A (reverse direction)
Patient
Wife
Daughter
Son
d
DL1000 Maker
Control
Patient
1kb
700bp
Patient
Forward
Reverse
Control
Forward
Reverse
Forward primer
AAAGGATTCAAACTTGCCCA
(629bp)
Reverse primer
TCCACAGTAACCAGGCAATG
WT
Exon 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
aa GDVINNGLWNM
375 385
(597bp)
Mut
Exon 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
aa GVCGRIRTX
375 382
e
Donor site scores: 82.83%
WT donor site 85.49%
WT: N ACAACTGGAGGTGATGTTATTAATAATGGTCTATGGAATATGgtacgtttgc
aa Thr Thr Gly Gly Asp Val Ile Asn Asn Gly Leu Trp Asn Met
372 373 374 375 376 377 378 379 380 381 382 383 384 385
Exon 10
Mut: N ACAACTGGAGgtgatgttattaataatggtctatggaatatggtacatttgc
aa Thr Thr Gly
372 373 374
Donor site scores: 82.83% Cryptic donor site
73.33% WT donor site
Exon 10
Legend exon intron

Fig. 1. A Chinese patient with pulmonary embolism and venous thromboembolism. (a) CT scanning and showed Pulmonary artery and its branch embolism in anterior inferior basal segment of right lower lobe. (b) Doppler ultrasound demonstrated thrombosis of the right femoral vein, superficial femoral vein and its branches. (c) *PROS1* IVS10 + 5G > A splice site mutation in DNA. (d) *PROS1* IVS10 + 5G > A splice site mutation in cDNA reverse transcribed from mRNA of PBMCs. A deletion of the last 32 nucleotides of exon 10 (NM_000313.3: c.1124_1155delGTGATGTTATTAATAATGGTCTATGGAATATG) which resulted in a frame-shift deletion (p.Asp376ValfsTer8) was found in the patient but not the healthy control subject. The forward primer was located in exon 8, and the reverse primer was located in exon 12. The amplicon of the wild type (WT) mRNA may produce a 629 bp fragment, while the amplicon of the mutant (Mut) potentially generated a 597 bp fragment. A stop codon occurred after 384th amino acid residue. (e) Splice site prediction with the Human Splicing Finder 3.0. Donor sites were disrupted as a score variation (between wild type and the mutant) > 10%, and in case of creating a donor site when a score > 80%.

mutation-negative thrombophilic families [6]. In this family, we identified non-classical *PROS1* IVS10 + 5G > A splice site mutation in the patient with PSD, which led to a deletion of the last 32 nucleotides of exon 10.

Eukaryotic genes are discontinuous, with protein-coding exons and intervening intronic sequences. The mRNA is synthesized as a precursor messenger RNA (pre-mRNA) in the nucleus during transcription. Pre-mRNA is transported to the cytoplasm where it serves as a template for protein biosynthesis after a series of processing steps. Pre-mRNA splicing was discovered 40 years ago, which is executed by a large and highly dynamic ribonucleoprotein complex named as the spliceosome. Alternative splicing is an essential step in the post-transcriptional regulation of gene expression, significantly expanding the functional proteome of eukaryotic organisms with limited gene numbers. Alternative pre-mRNA splicing occurs in > 95% of human genes, and the average human gene holds 8 exons and 7 introns, producing an average of three alternatively spliced mRNAs. Mechanisms of alternative splicing include RNA-RNA base-pairing interaction, RNA-protein interaction of splicing silencer or enhancer with regulatory sites, or chromatin-based effects which could determine the splicing pattern [7]. The GU dinucleotide of the 5′ splice site and AG dinucleotide of the 3′ splice site represent the classical spliceosome structure [8].

Assembly of the spliceosome depends strictly on the presence of the 5′ splice site, branch point sequence and 3′ splice site, which are conserved sequence elements of introns. Errors in pre-mRNA alternative splicing can lead to disease states. Mutations occurred in splice sites, intronic RNA regulatory silencer or enhancer, or in genes encoding splicing factors, could result in abnormal mRNA splicing, including deletion, insertion, or exon skipping [9]. Moreover, mutations adjacent to splice site or non-classical splice site have been also observed. *OCA2* IVS10-4A > G (NM_000275.2: c.1183-4A > G) was documented as rs10852218 in dbSNP database, and a high the minor allele frequency was observed in several databases, indicating that *OCA2* IVS10-4A > G mutation may be benign. Some non-classical splice site mutations were found to be pathogenic. *F8* IVS9 + 5G > C (NM_000132.3: c.1443 + 5G > C) was found to be associated with hemophilia A [10].

The non-classical *PROS1* IVS10 + 5G > A splice site mutation has been reported in several PSD patients [3,4], and it occurred in a CpG dinucleotide which may be a hotspot for mutation [3]. In previous studies, the effect of this non-classical splice site mutation on *PROS1* mRNA splicing remained unclear. P. H. Reitsma et al., analyzed PROS1 mRNA, however, they failed to detect the transcript from the mutated allele [4]. The signal of mutant was much lower than the wild type transcript observed in the electropherogram of the patient in the current study, indicating a small percentage of the mutant compared with the wild type transcript. A small percentage of the mutant might be missed by Sanger sequencing due to its detection limit of about 20% mutation, which might explain why P. H. Reitsma et al., failed to detect this mutant in cDNA. Moreover, according to Illumina bodyMap2 transcriptome project, PROS1 was highly expressed in liver but poorly expressed in white blood cells. Splicing is tissue dependent, therefore, as most PS is of liver origin, the analysis of RNA from PBMCs is only an indirect evidence of the potential effect of this mutation. The erroneously spliced mRNA product was unstable which may be degraded by nonsense-mediated decay (NMD) [9]. These reasons may partially explain why only mild levels of an aberrant RNA was detected according to the electropherogram, but low levels of PS detected in carriers (16–19%).

Low levels of PS have been widely found in patients with PSD harboring heterozygous nonsense or splice site mutation. In the study by Yolanda Espinosa-Parrilla et al., two unrelated PSD patients both carried heterozygous p.S293X nonsense mutation in exon 10, their activities of PS were < 12 and 16 respectively [11]. Less than 20% of serum PS activity was determined in a 58-year-old Korean male patient carrying p.K473X in exon 10 [12]. A Japanese patient carrying heterozygous protein S Sapporo 1 showed a PS activity of 13% [13]. These results suggest that PSD may be considered as an autosomal haploinsufficiency disorder [14]. Moreover, PS activity was influenced by several factors, including age, gender, oral anticoagulant treatment. In the study by Yolanda Espinosa-Parrilla et al., one patient (PS26) carrying heterozygous *PROS1* IVS10 + 5G > A mutation [11], had an activity of 39%. This patient developed VTE and superficial thrombophlebitis when he was 29 years old. In our study, the 46 years old proband developed VTE and PE. The proband's daughter harbor this mutation did not develop VTE when she was 28 years old.

In conclusion, this was the first report of *PROS1* IVS10 + 5G > A mutation in Chinese patient with PE and VTE partially due to PSD, and we further confirmed the effect of *PROS1* IVS10 + 5G > A mutation on *PROS1* mRNA splicing, which leads to the deletion of the last 32 nucleotides of exon 10, resulting in a frame-shift deletion and premature termination of PS (p.Asp376ValfsTer8). Our study suggests that *PROS1* IVS10 + 5G > A mutation may be the causal mutation of PSD.

Addendum

Yanjun Lu and Jun Yang were responsible for the design of the study. Xiong Wang was responsible for genetic analysis and writing the manuscript. Xu Wang was responsible for blood routine test. Ning Tang was responsible for coagulation tests.

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Compliance with ethical standards

The research was performed with an approval from the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Conflicts of interest

The authors declare that they have no conflict of interest.

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