A Thrombophilia Family With Protein S Deficiency Due to Protein Translation Disorders Caused By a Leu607Ser Heterozygous Mutation in PROS1

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Research

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Abstract

Background: Protein S deficiency (PSD) is an autosomal dominant hereditary disease. In 1984, familial PSD was reported to be prone to recurrent thrombosis. Follow-up studies have shown that heterozygous protein S (*PROS1*) mutations increase the risk of thrombosis. More than 300 *PROS1* mutations have been identified; among them, only a small number of mutations have been reported its possible mechanism to reduce plasma protein S (PS) levels. However, whether *PROS1* mutations affect protein structure and why it can induce PSD remains unknown.

Methods: The clinical phenotypes of the members of a family with thrombosis were collected. Their PS activity was measured using the coagulation method, whereas their protein C and antithrombin III activities were measured using methods such as the chromogenic substrate method. The proband and her parents were screened for the responsible mutation using second-generation whole exon sequencing, and the members of the family were verified for suspected mutations using Sanger sequencing. Mutant and wild type plasmids were constructed and transfected into HEK293T cells to detect the mRNA and protein expression of *PROS1*.

Results: In this family, the proband with venous thrombosis of both lower extremities, the proband's mother with pulmonary thrombosis and venous thrombosis of both lower extremities, and the proband's younger brother had significantly lower PS activity and carried a PROS1 c. 1820T > C:p.Leu607Ser heterozygous mutation (NM_000313.3). However, no such mutations were found in family members with normal PS activity. The PS expression in the cell lysate and supernatant of the Leu607Ser mutant cells decreased, while mRNA expression increased. Immunofluorescence localization showed that there was no significant difference in protein localization before and after mutation.

Conclusions: The analysis of family phenotype, gene association, and cell function tests suggest that the PROS Leu607Ser heterozygous mutation may be a pathogenic mutation. Serine substitution causes structural instability of the entire protein. These data indicate that impaired PS translation and synthesis or possible secretion impairment is the main pathogenesis of this family with hereditary PSD and thrombophilia.

Background

Protein S (PS) is a vitamin K-dependent plasma glycoprotein that is mainly synthesized by hepatocytes or macrophages [1]. Forty percent of PS is free and has anticoagulant activity, while 60% of PS is bound to C4b and has no activity [2]. On the one hand, PS exerts an anticoagulant effect mainly by serving as a cofactor of activated protein C (APC) to promote the inactivation of factor V (FV) a and FVIIIa [3]. On the other hand, PS also serves as a cofactor of tissue factor pathway inhibitor (TFPI), which inhibits the activity of tissue factors by promoting the binding interaction of TFPI and FXa [4]. Hereditary protein S deficiency (PSD) is an autosomal dominant hereditary disease, which may be caused by genetic and acquired factors [5]. It is classified into three subtypes: Type I (total PS, free PS levels, and PS activity are

decreased), type II (total PS and free PS levels are normal, but PS activity is decreased), and type III (total PS level is normal, but free PS level and PS activity are decreased) [6]. There is no significant difference among these three types of clinical manifestations, which are only identified by laboratory testing; 95% of patients with PSD develop type I and type III PSD [7].

As of September 6, 2021, there are more than 360 mutations in PSD-related genes in the Human Gene Mutation Database (HGMD) (http://www.hgmd.org). There are 276 types of missense/nonsense, 48 types of splicing, 4 types of regulatory, 54 types of small deletions, 25 types of small insertions, 6 types of small indels, 28 types of gross deletions, 7 types of gross insertions, as well as complex repeats that have not yet been identified. The most common causes of PSD are missense or nonsense substitutions, followed by splice site mutations, small or large repeats, insertions, or deletions [8]. The main clinical manifestations of most patients with heterozygous mutations in the protein S gene (*PROS1)* are lower extremity deep venous thrombosis and pulmonary embolism [9]. *PROS1* mutations are associated with an increased risk of venous thrombosis [10], and some reports suggest that *PROS1* variants increase the risk of arterial embolism, such as cerebral infarction and myocardial infarction [11]. About half of patients with PSD develop symptoms before the age of 55, while some of them have no complications for the rest of their lives [12]. In the past, we detected a new mutation in *PROS1* in a family prone to thrombosis, which had not been previously reported. In this study, we discuss the pathogenicity and pathogenesis of this mutation.

Methods And Materials

Research subjects

The 16-year-old female proband (III5), of Han nationality, complained of "swelling and pain in the left lower limb for 3 days". She was in good health and had no bad lifestyle-related habits, such as, smoking, drinking etc. Among the family members, her mother (II8) had a history of bilateral deep venous thrombosis of the lower extremities and pulmonary embolism, and her parents were from non-consanguineous marriages. Physical examination showed that the left lower limb of the proband had edema, especially on the dorsal foot, shank, and thigh. There were no obvious varicose veins, hyperpigmentation, skin ulceration, palpable nodules, or deep vein tenderness with a positive Homan’s sign. The circumference of both lower limbs was measured and was as follows: 15 cm above the left patella, 44 cm; 15 cm above the right patella, 39 cm; 15 cm below the left patella, 39 cm; and 15 cm below the right patella, 36 cm. The rest of the physical examination showed no obvious abnormalities. Relevant examinations were performed after hospital admission.

At the age of 39, II8 had complained of "distension and pain of the left lower limb for 2 days" in another hospital. She was in good health and had no special bad habits. Physical examination revealed swelling of the left lower limb. She was diagnosed with "deep venous thrombosis of the left lower limb" and was treated with anticoagulation and thrombolysis. After that, she improved and was discharged from the hospital and took anticoagulants regularly for a long time. A year ago, she visited the hospital again due

to "sudden chest pain with loss of consciousness" and was diagnosed with "pulmonary embolism." The father of II8 (I1) and her two older sisters (II 3 and 4) all died of pulmonary embolism and deep vein thrombosis (DVT) of the lower limbs. To date, no thrombosis has been found in other family members.

Methods

Clinical phenotype detection Clinical phenotypes and clinical biochemical indicators were collected from the proband and her related family members. Clinical biochemical indexes included PS activity, as measured using the coagulation method and the activity of protein C (PC) and antithrombin III (AT-III), as measured using the chromogenic substrate method, blood routine, coagulation function, and biochemistry.

Extraction of genomic DNA Peripheral blood (8 mL) of the proband and peripheral blood (2 mL) of each family member were collected in ethylenediaminetetraacetic acid anticoagulant tubes, and genomic DNA of the proband and her family members was extracted using the QIAGEN DNA Blood Mini Kit (Cat# 51106, QIAGEN Co., Ltd., Shanghai, China).

Location and screening strategy of mutant genes The TargetSeq® liquid probe hybridization and capture technique independently developed by Igen iGeneTech® (Beijing, China) was used to establish a genomic DNA library and capture the promoter and exon regions (16.06 Mbp) of 5,081 genes related to genetic diseases. Paired end 150 bp sequencing was performed using the Illumina X10 or NovaSeq 6000 platform. The captured target genes were *PROS1* and Serpin family C member 1 (*SERPINC1*). Based on the results of BAM alignment with the genome reference sequence, single-nucleotide variants and indels in the samtools, GATK, and ANNOVAR sequencing results were used to remove the variation sites with intermediate frequency higher than 0.01 in ExAC, gnomAD, iGeneTechDB (local database with more than 10,000 samples), benign and likely benign mutations in ClinVar, and synonymous_variant mutations in the Human Genome Variation Society. Combined with the exon sequencing data of the parents, the sources of mutation were annotated and divided into three types: those from the father, from the mother, and suspected to be new mutations. The Hemostasis Thrombosis Expert Panel of the OMIM Phenotypic Series-PS188050 and CLINGENE were used to search for genes. Mutations from the father were excluded (the mutations from the mother and the suspected new mutations were retained), and two mutations in *SERPINC1* and *PROS1* were obtained. Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen-2, http://genetics. Bwh.harvard.edu/ppH2/) and Mutation Taster (http://mutationtaster.org/) were used to predict the pathogenicity and harmfulness of the mutations. The upper and downstream positions of the sequence of the target mutation site were designed using Premier 5.0, and the target area was amplified. The corresponding suspected pathogenic mutations were verified by Sanger sequencing using the ABI3500Dx platform. The amplified fragment length of c. 1820T > C:p, the Leu607Ser sequence of the mutation point in *PROS1* (NM_000313.3), was 498 bp. The primers F: CTGGCTGGGATAGCCAAATGA and R: CTTGCTTATATTGAATCTTTGCTCTGC were used for amplification (melting temperature, 62.5 °C). The amplified fragment length of c.883G > A:p, the Val295Met sequence of *SERPINC1* (NM_000488.3), was 407 bp. The primers F:

CTTGCAGCTGCTCCTTCAAACT and R: TGTCTTGTGTCAATAACTATCCTCCTA were used for amplification (melting temperature, 61 °C). Synbio Technologies Co., Ltd. (Suzhou, China) synthesized all primers.

Construction and identification of *PROS1* wild type (WT) and p.Leu607Ser mutant plasmids

The plasmid synthesis scheme is shown in Fig. 4a. pcDNA3.1-3×Flag-C was used as the expression vector to synthesize *PROS1* with a KpnI/XhoI cleavage site. The WT plasmid 1 (PC DNA 3.1-ProS1 WT-3 × Flag-C) and the mutant plasmid 2 (pcDNA3.1-PROS1mut-3×Flag-C) were constructed, both with a KpnI/XhoI restriction enzyme site. The mutant plasmid 2 contained the 1820T>C mutation in *PROS1*. Target genes were amplified and sequenced. The cloning of *PROS1* (WT) and *PROS1* (1820T > C) and the synthesis of related polymerase chain reaction (PCR) primers were performed by Wuhan Gene Create Biological Engineering Co. Ltd(Wuhan, China).

Cell transfection

HEK293T cells were digested and collected using trypsin, and the cells were placed into a 10 cm petri dish at a density of $1–2 \times 10^7$ cells/plate in an appropriate complete culture medium. After adhesion, the total area of the cells reached 80%–90% confluence. According to the conditions of cell adhesion, cells were incubated at 37 °C in an incubator containing 5% CO_2 for 8–24 h, and transient transfection was started after the cells were completely adhered. According to the instructions for TurboFect (R0531, Thermo, Massachusetts, USA), TurboFect-DNA Mix was prepared and mixed with DNA plasmids (10 μg/*PROS1* WT, mutant, or control plasmid + 5 μg green fluorescent protein [GFP]) and 30 μL TurboFect in 1000 μL Opti-Medium. After incubation at room temperature for 15 min, TurboFect-DNA Mix was added to the petri dish. After 12 h, the complete medium was changed, and HEK293T cells were cultured for 48 h. Cells were observed to be in good condition by microscopy and the culture medium was collected for further evaluation.

Quantitative real-time (qRT)-PCR detection

HEK293T total RNA was extracted according to the TriPure Isolation Reagent kit (11667165001, Roche, Shanghai, China) , and the difference in the *PROS1* transcription levels was detected by reverse transcription and qRT-PCR. The first chain of cDNA was synthesized according to HiFiScript (CW2020M, CWBIO, Beijing, China) . The reaction system contained 2.5 mM dNTP Mix, 4 μL; primer mix, 2 μL (primers in Table 1); RNA Template, 7 μL; 5× RT Buffer, 4 μL; 1× dithiothreitol, 0.1 M, 2 μL; 10 mM HiFiScript, 200 U/μL; and RNase-free water, 20 μL. After mixing the liquid using a vortexer, the tube was centrifuged for a short time. The product was incubated at 42 °C for 50 min and at 85 °C for 5 min. The cDNA obtained by reverse transcription was diluted 20-fold, and 40 RT-qPCR cycles were performed in a Roche LightCycler 480 (Roche, Beijing, China).

Western blot detection

HEK293T cells were cultured, lysed, total protein was extracted, and PROS1 expression was detected. Protein samples were separated using electrophoresis and then wet transferred to a polyvinylidene fluoride (PVDF) membrane, soaked in 5% skim milk prepared in Tris-buffered saline with 0.1% Tween® 20 (TBST), and sealed at room temperature for 1 h. Next, the membrane was washed once and anti-protein S antibody (97387, Abcam, UK, 1: 500) or actin antibody (ab8227, Abcam, UK, 1: 5000) was added. The Flag antibody (F3165, Sigma, USA, 1:500), diluted with 5% bovine serum albumin (BSA), was added to the membrane overnight at 4 °C, and the membrane was washed thrice. Horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit IgG, 1:2,000 or goat anti-mouse IgG 1:2,000, diluted with 5% BSA, ab6721 and ab6789, respectively, Abcam, UK) were added to the membrane and then incubated in a shaker at room temperature for 1 h. The PVDF membrane was washed with TBST five times and with ddH_2O once before exposure.

Enzyme linked immunosorbent assay (ELISA) of PROS1 in HEK293T cell lysates and cell supernatants

According to the instructions of the Human Protein S ELISA Kit (ab190808, Abcam, UK), the working standard liquid was prepared, and PROS1 expression in HEK293T cell lysates and cell supernatant was detected. A microplate reader (Varioskan Lux, Thermo, Massachusetts, USA) was used to measure the optical density at 450 nm immediately after the substrate solution was added to stop the reaction. A standard curve was created and PROS1 levels in the sample were calculated.

Immunofluorescence localization experiment

After being fixed, permeabilized, and blocked, the transfected HEK293T cells were incubated at 4 °C overnight with the PROS1 primary antibody (diluted 1:200). The transfected HEK293T cells were rinsed with phosphate-buffered saline (PBS) thrice, the fluorescent secondary antibody (diluted 1:500) was added and incubated at room temperature in the dark for 2 h, rinsed with PBS thrice, and stained with 4',6-diamidino-2-phenylindole. The transfected HEK293T cells were incubated at room temperature for 5 min and rinsed twice with 1× PBS for 3 min each time. A laser confocal microscope (Nikon A1, Shanghai, China) was used to capture images.

Statistics

Experimental data were statistically analyzed using GraphPad Prism 6.02. An unpaired *t*-test was used to compare the two groups. The mean value was expressed as the mean ± standard error of the mean (SEM), and $p < 0.05$ indicated that the difference was statistically significant.

Results

Clinical phenotypes

The 16-year-old proband (III5) was examined for blood coagulation function after admission (Table 2). The indices related to blood coagulation increased significantly, including the prothrombin time (PT, 13.3

s), international normalized ratio (INR, 1.17), fibrinogen degradation product (FDP, 57.2 μg/mL), and D-dimer levels (25.8 mg/L). Color Doppler ultrasound of the lower limb vein showed thrombosis of the left external iliac vein and deep vein of the left lower limb. Digital subtraction angiography (Figure 1) showed a filling defect in the distal left superficial femoral vein without development and a filling defect of the distal left wall of the left inferior vena cava, indicating thrombosis. PS activity was < 16% and was significantly decreased, while the PC and AT-III activities were normal; thus, it was considered asthrombosis caused by PSD. The mother of the proband (II8), 42 years old, had a history of recurrent venous thrombosis. For the first time, when lower limb swelling and pain occurred, coagulation function was checked (Table 2), showing that the indices related to blood coagulation were significantly increased. PT was 14.7 s, INR was 1.37, activated partial thromboplastin time was 101.6 s, and thrombin time (TT) was 60 s. Color Doppler ultrasound showed that the inner diameter of the left common femoral vein, superficial femoral vein, deep femoral vein, popliteal vein, and peroneal vein were widened, and the left common femoral vein had a solid echo. Color Doppler flow imaging (CDFI) showed a small number of short-strip blood flow signals in the upper segment of the superficial femoral vein, suggesting DVT of the left lower extremities. Computed tomographic angiography (CTA) (Figure 2 f–n) of the lower limbs showed a low-density filling defect in the common iliac vein, external iliac vein, femoral vein, and popliteal vein lumen, a blurred deep vein of the lower limb, and swelling of the soft tissue of the left lower limb. She was considered to have “deep venous thrombosis of the left lower limb.” The second time, 2 years later, when chest pain occurred, CTA (Figure 2a–e) showed a low-density filling defect in the distal left and right pulmonary arteries and bilateral pulmonary artery branches, suggesting pulmonary embolism. Color Doppler ultrasound showed hypoechoic filling in the lower part of the left superficial femoral artery and left popliteal vein. CDFI showed a small number of irregular blood flow signals, suggesting that the deep vein of the left lower extremity and the right popliteal vein were partly recanalized after thrombosis. PS activity was less than 16% and the activity of AT-III and PC was normal, so it was suspected that PSD caused thrombosis in II8 many times. The 13-year-old younger brother of the proband (III6) had no history of thrombosis. His PS activity was 16.7% and the PC and AT-III activities were normal; thus, he was diagnosed with PSD. His TT was 30.9 s, which was obviously higher than the normal value. The family was investigated, and a pedigree map of the genetic family was drawn (Figure 3a).

Screening for a thrombophilia gene mutation

The members of the family were analyzed by whole exon sequencing, and two mutation sites (Figure 3b–e) were identified in proband III5. One was a heterozygous mutation of exon 14 in *PROS1* (NM_000313.3): c.1820T>C(p.Leu607Ser); the transformation from TTG to TCG was not recorded in the ClinVar and HGMD databases. Thus, this is a newly discovered mutation, and its pathogenicity is unclear.

The other was a heterozygous mutation of exon 3 in *SERPINC1* (NM_000488.3): c.883G > A (p.Val295Met) (rs201381904). This mutation has not been recorded in the ClinVar and HGMD databases, and its pathogenicity is not clear. According to SIFT [13] and PolyPhen-2 [14] scores, the *PROS1* mutation SIFT score is 0, PolyPhen-2 score is 1, and Mutation Tester [15] predicts that protein function is

moderately affected. The *SERPINC1* mutation SIFT score was 0.036, and the PolyPhen-2 score was 0.996. The lower the SIFT score, the greater the harm and the closer the PolyPhen-2 score to 1, the stronger the pathogenicity. The *PROS1* L607S heterozygous mutation and *SERPINC1* V295M heterozygous mutation were identified in II8, and a *PROS1* L607S heterozygous mutation was also identified in III6. Except for the heterozygous mutation V295M carried by II1, there were no L607S and V295M mutations in other family members.

Cloning of *PROS1* WT and p.Leu607Ser gene mutations

The *PROS1* WT and *PROS1*/p.Leu607Ser cloning and eukaryotic expression vectors were successfully constructed. Fragments of WT *PROS1* and mutant *PROS1*/pLeu607Ser digested by KpnI/XhoI were approximately 1138 bp, which was consistent with plasmid design. The constructed vectors were verified by sequencing and transfected successfully into HEK293T cells (Figure 4 c).

Localization of WT PROS1 and its mutants in cells

The localization of WT and mutant PROS1 was detected by immunofluorescence (Figure 5), which showed that PROS1 was distributed in the interior of the cells. Because the protein is a secretory protein, there was no significant difference in intracellular fluorescence localization before and after introduction of the *PROS1* 1820T > C mutation.

Expression of WT *PROS1* and its mutants in HEK293T cells

The relative mRNA expression of the WT (*PROS1*-WT) and p.Leu607Ser mutant (*PROS1*-MUT) *PROS1* in HEK293T cells was detected using qRT-PCR. The difference between the *PROS1* mRNA expression groups was compared to that of actin (Figure 6b) as an internal reference when there was no significant difference in transfection efficiency among groups (Figure 6a). *PROS1* mRNA expression was significantly upregulated with the *PROS1* 1820T > C mutation ($p < 0.05$). GFP was used as an external reference (Figure 6c) to compare the difference between the *PROS1* mRNA expression between groups. Again *PROS1* mRNA expression was significantly upregulated with the *PROS1* 1820T>C mutation ($p < 0.01$). PS expression (Figure 6d) in the cell supernatant and lysate was detected by western blotting. PROS1 expression was significantly downregulated with the mutation. At the same time, PROS1 expression in the supernatant of the cell culture medium and cell lysate was detected using ELISA (Figure 6e, f). PROS1 expression levels in the culture medium supernatant and cell lysate of the p.Leu607Ser mutant group was significantly lower than that of the WT group, which was consistent with the results of western blotting.

Discussion

In this study, the proband (III5) was a 16-year-old young woman who developed deep venous thrombosis of the left lower limb without exposure to other risk factors, which was suspected to be caused by familial heredity. Therefore, the whole exon of III5 was sequenced using next generation sequencing, and a

suspicious variation in two genes was identified. One was *SERPINC1* c.883G > A (p.Val295Met), resulting in the substitution of methionine for valine located at 1q25.1, and containing nine exons, which encoded AT-III. The mutation of a single base leads to AT-III deficiency, which causes the plasma AT-III levels and activity of patients to obviously decrease[16]. Hereditary AT deficiency is autosomal dominant, and thrombosis caused by hereditary AT deficiency is usually more serious than *PROS1* and *PROC* mutations [17]. The AT-III plasma levels in the members of this family were normal, which ruled out the diagnosis of hereditary AT deficiency. The other mutation was *PROS1* c. 1820T > C (p.Leu607Ser). The transformation from TTG to TCG resulted in the replacement of leucine with serine. *PROS1* is located near the centromere of chromosome 3 (3q11.1); it contains 15 exons and encodes PS [18]. From the N-terminal to the C-terminal, there is a γ-carboxyl glutamate domain, a region sensitive to cleavage by thrombin, four domains homologous to epidermal growth factor, and a region homologous to sex hormone binding globulin (SHBG). SHBG contains two tandem laminin G regions (LG1 and LG2) [19]. Mutations in *PROS1* are a risk factor for thrombosis in Asian populations and repeated spontaneous DVT and pulmonary embolism without obvious reasons are the most common symptoms [20]. Whether individuals with *PROS1* mutations have thrombosis greatly depends on the interaction between genes and the interaction between genes and the environment. However, compared to individuals without gene mutations, the risk of thrombosis with gene mutations is 2–11 times higher [21]. Mutations in *PROS1* lead to decreased PS levels and activity in patients and lead to PSD, which reduces the ability of auxiliary APC to inactivate FVa and FVIIa, resulting in thrombosis. For example, a missense *PROS1* mutation (Gly222Arg) has been identified in a patient with pulmonary embolism, which causes PS activity to decrease to 5.0% [22].The PS activity of several codon mutations near L607, such as Ser627fs, Ser627 ins101fsX34 (acc HGMD nomenclature), p.Ala536Val, p.Asn583His, p.Thr617Ile, p.Asp624His, and p.Cys666Ser (acc HGVS nomenclature) are all less than 40%, and the lowest is 12%, suggesting that mutation of the corresponding domain causes serious functional defects [23]. The diagnosis of hereditary PSD is generally based on clinical manifestations, plasma PS levels, activity detection, and gene detection[24].

The elder sister and father of II8 died of pulmonary embolism, so there was an obvious genetic tendency in this family. The L607S mutation in III5 and III6 was derived from II8, which was consistent with autosomal dominant hereditary disease and could be diagnosed as hereditary PSD. Although not every patient with PSD has a clinical phenotype, similar to III6, it will obviously increase the risk of thrombosis, especially in patients with heterozygous mutations and PS activity less than 30% [25]. The occurrence of clinical phenotypes is related to age, sex, and mutation type [26]. There are more male patients with hereditary PSD than female patients with hereditary PSD, but the peak age in females is younger, which is due to the influence of hormones and risk factors, such as trauma, surgery, and oral contraceptives [27]. At present, III6 is a 12-year-old male, but thrombosis is likely to occur with increased age. Coagulation function and venous ultrasound examination should be regularly followed up, and anticoagulant therapy may be administered when appropriate. If *PROS1* occurs as a homozygous mutation, the prognosis is poor and the child may die of fulminant purple spot caused by severe PSD in the neonatal period [28]. Similar to the results of an animal experiment, explosive bleeding in

PROS1 -/-homozygous mice is observed in mice with *PROS1* knockout at the embryonic stage. *PROS1-/+* heterozygous mice survive to adulthood, but different degrees of vascular injury and dysplasia are observed [29]. At the same time, the level of PROS1 and the activity of auxiliary APC are detected, which are significantly lower than those of WT mice [29]. To verify whether the pathogenicity of *PROS1* L607S was consistent with the phenotype observed, plasmids carrying the L607S mutation or WT were constructed and transfected into HEK293T cells. After the *PROS1* 1820T > C mutation was introduced, *PROS1* mRNA expression was obviously upregulated, while PROS1 expression was obviously downregulated in the cell lysate and supernatant. Immunofluorescence localization showed that the protein was distributed in the cell interior, which indicated that the missense mutation did not affect *PROS1* mRNA expression, but PS expression and secretion was obviously decreased.

This may be because the mutated residues affect the level of translation and post-translation modification, resulting in disordered protein processing and secretion, which is the main molecular disease mechanism of most missense and other mutations in genetic diseases [30]. In humans and other mammals [31, 32], a comparison between Leu607 of PS and the adjacent flanking structures of PS show that this site is highly conserved (Figure 7). Further analysis of other substitutions was performed on the bases at this site, and Alamut Visual Plus software was used to construct the different variant bases. The substitution of Leu by Trp at codon 607 forms a termination codon. The harmfulness of L607Y was consistent with the Mutation Taster analysis and L607S prediction; L607Y was harmful and moderately affected protein function. In addition, L607* might produce a truncated protein, which was predicted to cause disease. Therefore, it was concluded that protein synthesis and secretion might be affected by the Leu607 conserved site. The Leu607 conserved site plays a key role in the structure and conformation of SHBG and may be important in the PS anticoagulant effect; about half of the *PROS1* mutations in the LamG domain involve the acquisition and loss of residues with unique physical and chemical properties, such as cysteine, proline, and glycine, which directly affect PS function [33]. Because most of the mutant residues are hydrophobic, changes in these residues may affect protein folding and secretion [34].

In a PSD family, COS-7 cells were transfected with plasmids containing the D38Y and P626L mutant genes; the PS level and activity of D38Y and P626L mutants is significantly decreased [35], which is consistent with the results of this study. In this study, the hydrophobic amino acid Leu at position 607 was replaced by the polar neutral amino acid Ser. While Ser itself has no charge, its side chain has one more free hydroxyl group than Leu in structure, which is easily phosphorylated by protein kinase. Phosphorylation is the most important post-translational modification and has the greatest effect on the local and overall structural changes in proteins; most phosphorylation occurs on serine residues [36]. The phosphate group formed after phosphorylation of the mutated Ser607 may form hydrogen bonds or salt bridges with the adjacent Lys609, making the local structure compact, which may change the overall conformation of the protein and the interaction between proteins to regulate function [37]. The mutant (https://swissmodel.expasy.org/interactive/7J8pZH/models/) and WT PROS1 (https://swissmodel.expasy.org/interactive/HxBw4h/models/) homologous proteins were

constructed using the Swiss Model [38], and the characteristics of the advanced structure were observed. The number of amino acid residues in the Ca^{2+} region of the mutant protein was one less than that of the WT protein, which might be due to the change in local protein structure caused by Ser phosphorylation, leading to a decrease in the binding of PS to other proteins, decreased PS activity, and anticoagulant dysfunction. Ca^{2+} regulates the binding of the C-terminal SHBG region of bound PS to C4 binding protein (C4BP)[39]. Both LG1 and LG2 are involved in PS binding to C4BP, showing anticoagulant activity independent of APC [40]. If PS residues Lys423, Lys427, and Lys429 are replaced by other polar amino acids, the binding force between PS and C4BP is reduced by 5–10-fold. Insertion of alanine at position 611 leads to the loss of binding to C4BP [41], which leads to a decrease in anticoagulant function. The anticoagulant activity of free PS through the tissue factor pathway inhibitor (TFPI) is also through the combination of SHBG and TFPI, which further promotes the interaction between TPFI and FX a, thus inhibiting the activity of tissue factor [2]. LG1 and LG2 are necessary for the binding of SHBG and TFPI, but LG1 plays a major role [42]. A R474C mutation leads to PS secretion disorder and intracellular degradation, resulting in type I PSD[43]. By constructing mutant transfected cells, the R474C mutant reduces PS secretion by eight-fold and shortens the half-life of radioactive markers [43]. This might be due to a change in local protein folding. Protein misfolding leads to stagnation in Golgi cells, while unfolded proteins remain in the endoplasmic reticulum cavity for a long time due to molecular chaperone activity. Then, the mechanism of endoplasmic reticulum-associated protein degradation is initiated, which leads to the decomposition of related proteins in cells [44].

Conclusions

In this study, a heterozygous mutation of *PROS1*, c.1820T > C:p.Leu607Ser, was identified as a pathogenic mutation that caused disorderly PS translation, synthesis, and secretion or intracellular degradation, and finally led to a decrease in PS levels and activity, resulting in PSD. Heterozygous mutation of *PROS1* c.1820T > C:pLeu607Ser was familial.

Abbreviations

PS: protein S

PROS1: protein S gene

PSD: protein S deficiency

SERPINC1: Serpin family C member 1

TFPI: tissue factor pathway inhibitor

APC: activated protein C

qRT-PCR: quantitative real-time-polymerase chain reaction

DVT: deep vein thrombosis

PC: protein C

AT-III: antithrombin III

WT: wild type

PVDF: polyvinylidene fluoride

BSA: bovine serum albumin

ELISA: Enzyme linked immunosorbent assay

PBS: phosphate-buffered saline

PT: prothrombin time

INR: international normalized ratio

FDP: fibrinogen degradation product

TT: thrombin time

CDFI: Color Doppler flow imaging

CTA: Computed tomographic angiography

SHBG: sex hormone binding globulin

LG: laminin G regions

C4BP: C4 binding protein

Declarations

Ethics approval and consent to participate: All procedures were performed in accordance to the tenets of the Declaration of Helsinki and the study was approved by the Ethics Committee of Fujian Provincial Hospital, Fuzhou, China. All participants and legal guardians of the minors involved in the present study provided written informed consent.

Consent for publication: Each subject has a signed consent form.

Availability of data and materials: The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Competing interests: The authors have no conflicts of interest to declare.

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Tables

Table 1 Primers for qRT-PCR

hPROS1 qRT F	CCCGGAAACGGATTATTTTT
hPROS1 qRT R	CTCCTTGCCAACCTGGTTTA
hGAPDH F	agaaggctggggctcatttg
hGAPDH R	aggggccatccacagtcttc
CopGFP qRT F	aggacagcgtgatcttcacc
CopGFP qRT R	cttgaagtgcatgtggctgt

Table 2 Coagulation function indexs of proband and family members in hereditary protein S deficiency family

Items	Propositus (Ⅱ5)	Father (Ⅱ7)	Mother (Ⅱ8)	Brother (Ⅱ6)	Member (II1)	Member (II6)	Member (Ⅱ10)	reference value
PT (s)	13.3*	11.2	14.7*	12.3	9.8	11.9	12.1	9.9-12.9
INR	1.17*	0.89	1.37*	1.07	0.92	1.02	1.11	0.79-1.12
APTT (s)	27.9	26.4	101.6*	32	27.1	24.3	23.9	23.3-32.5
TT (s)	15.5	15.8	60*	30.9*	17.1	16.4	19.2	14-21
Fg (g/L)	2.57	2.32	0.9^	1.9	2.41	2.79	2.34	1.8-3.5
FDP （mg/L）	57.2*	-	-	-	-	-	-	0-5
D-dimer	25.8*	0.24	13.2*	15.5*	0.23	0.01	0.01	0-0.55
PS(%)	<16^	84.4	<16^	16.7^	102.7	97.4	112.3	Male（75-130 Female（52-118
PC(%)	66.9	90.1	100.2	60.2	113.4	124.2	98.1	70-140
AT-Ⅲ(%)	92	107.6	97.4	85.9	121.2	109.1	97.1	75-125

Note: PT :prothrombin time; INR: international normalized ratio; APTT: activated partial thromboplastin time; TT: thrombin time; Fg: fibrinogen; FDP: fibrin degradation products; PS: protein S; PC: protein C; AT-Ⅲ: antithrombin Ⅲ; *: represents a significant increase; ^: represents a significant decrease.

Figures

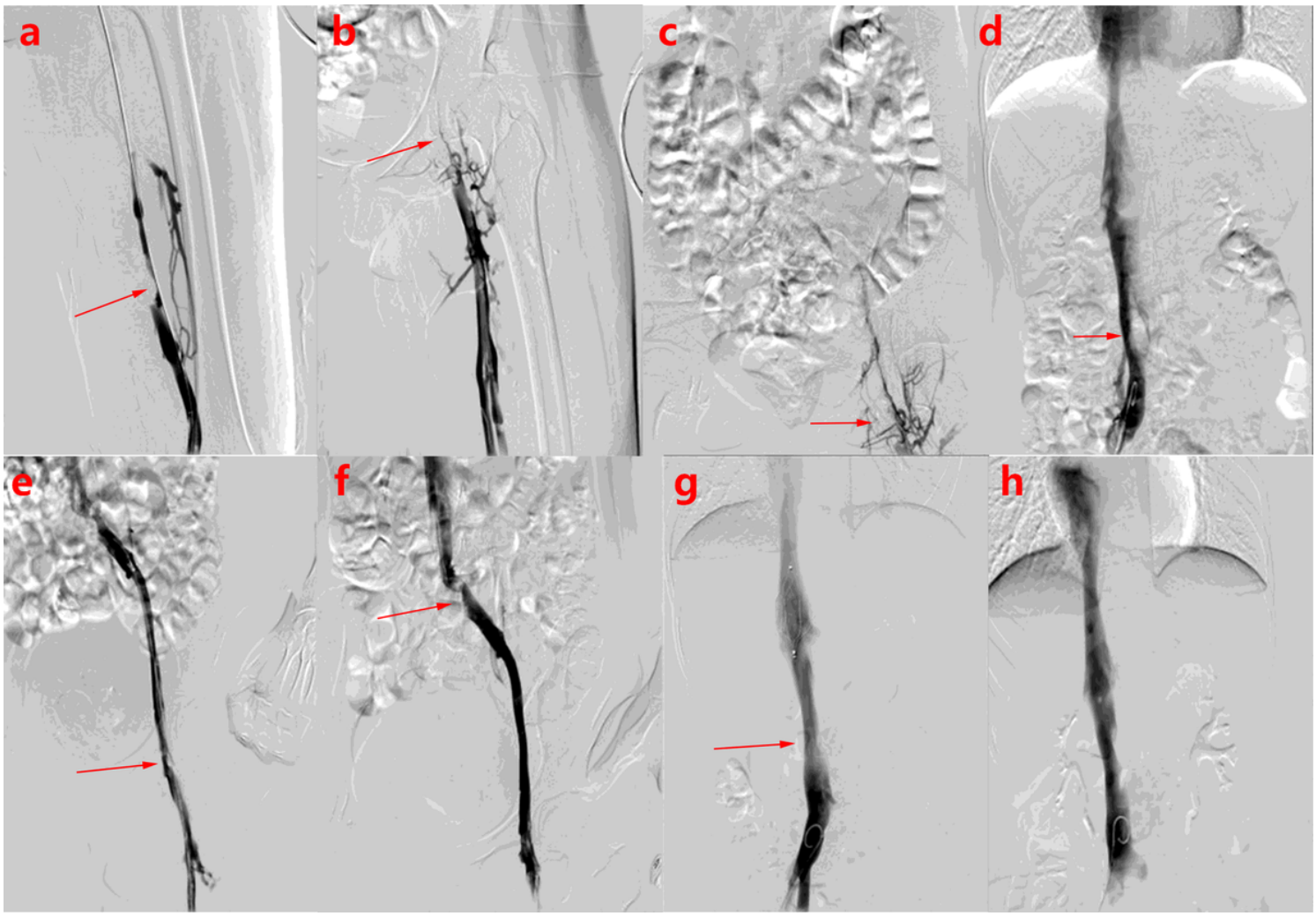


Figure 1

Digital subtraction angiography (DSA) of the proband (III5). (a) Proximal angiography of the left superficial femoral vein shows a filling defect without development. (b) The left iliac vein is not developed and some collateral vessels are observed. (c) The distal end of the inferior vena cava shows a filling defect and thrombosis in the left side wall. (d) After thrombolysis with 2 million units of urokinase, the left superficial femoral vein (e), left iliac vein (f), and left wall of the distal inferior vena cava (g) show no filling defect, and the thrombus is dissolved and absorbed. Angiographic picture after removal of the inferior vena cava filter (h).

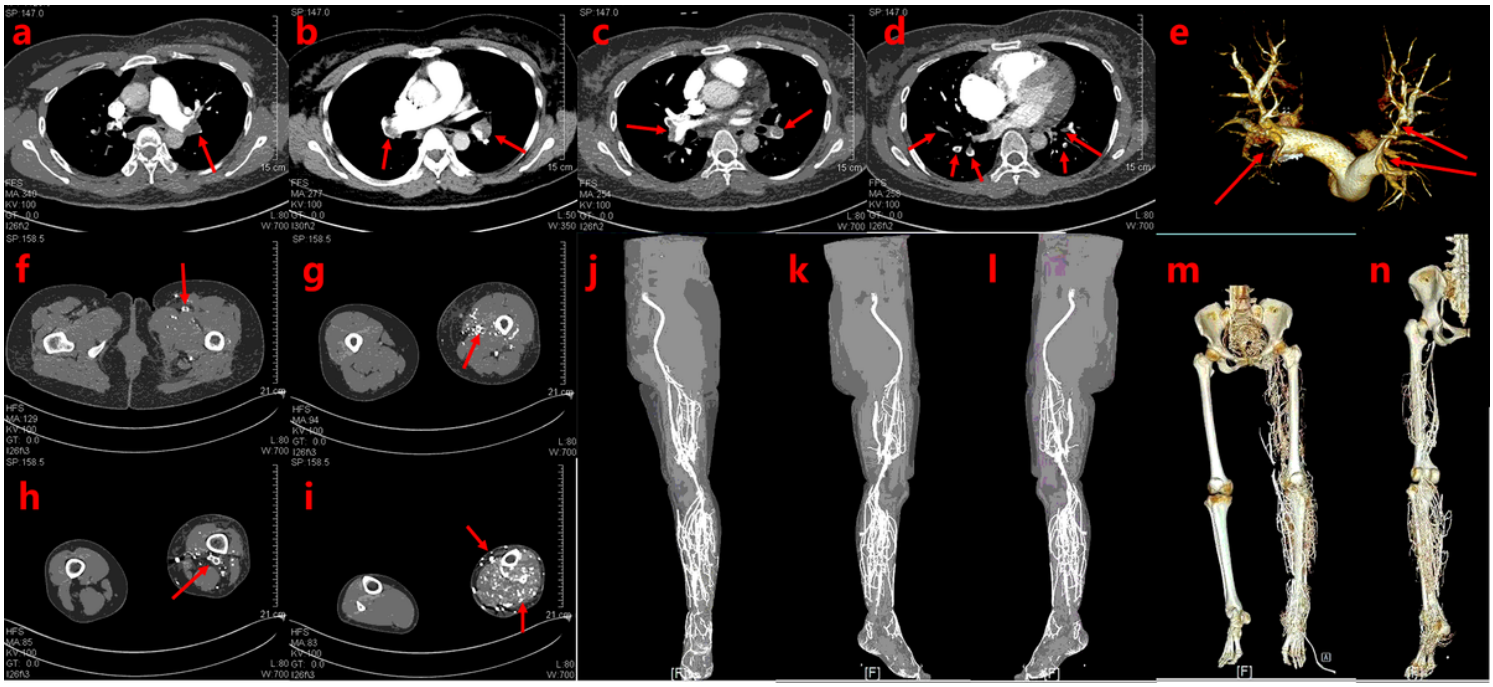


Figure 2

Computed tomographic angiography (CTA) of the mother of the proband (II8). CTA shows a low density filling defect in the left common iliac vein, external iliac vein, femoral vein, and popliteal vein. The contrast medium is narrowed at the local edge and passes through in a complete ring, surrounded by multiple collateral vessels with local reticulation. Multiple tortuous vessels are observed in the left leg, the deep veins of the lower extremities are not clearly displayed, and the soft tissue of the left lower extremities is swollen. Therefore, deep venous thrombosis of the lower extremities (Figure 4f-n) is considered. Low-density filling defects are observed locally in the distal end of the left and right pulmonary arteries and in the branches of both pulmonary arteries, suggesting pulmonary embolism (Figure 4a-e).

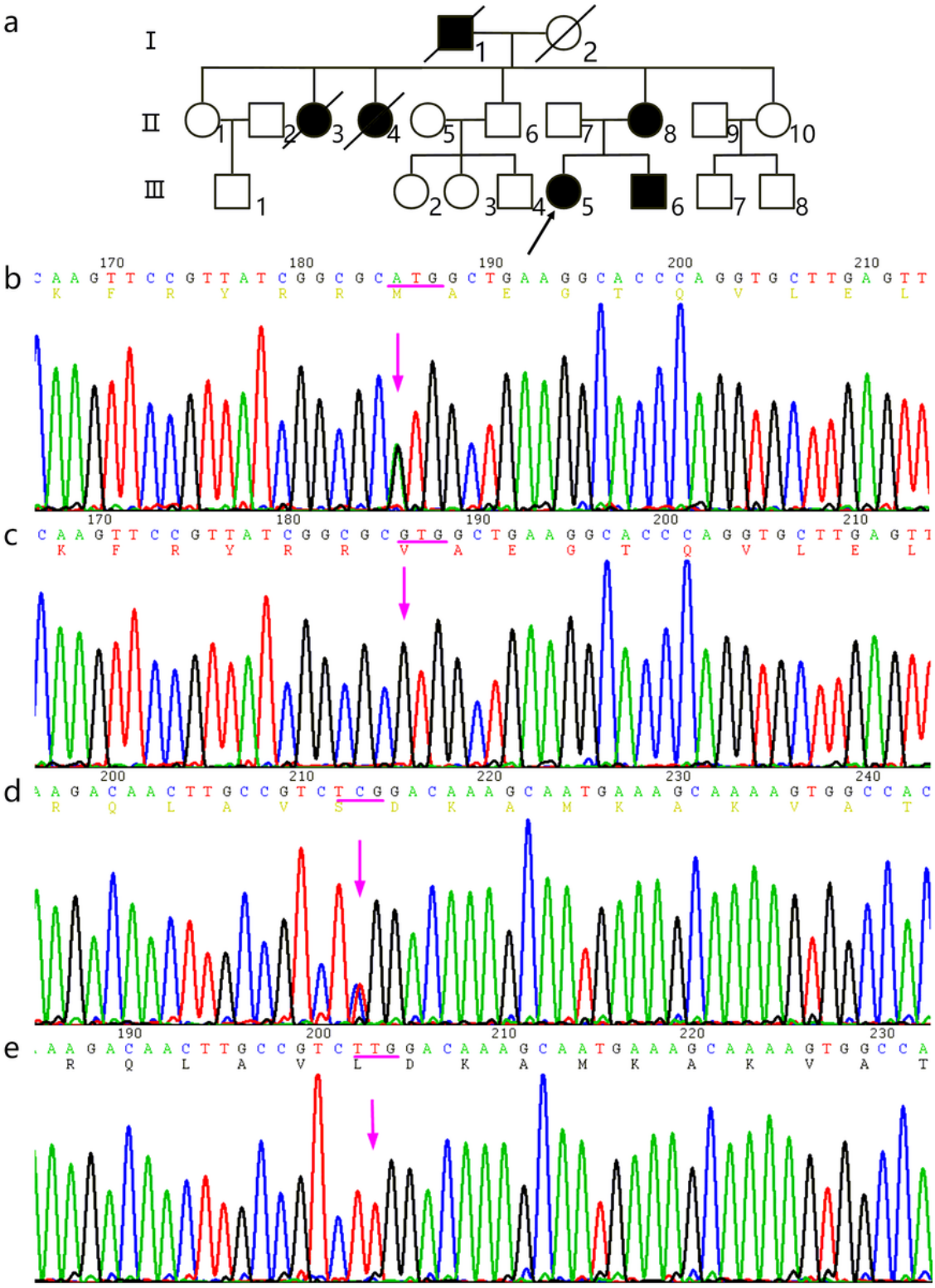


Figure 3

Depiction of the protein S (PROS1) and Serpin family C member 1 (SERPINC1) mutations. (a) In the family map of hereditary protein S deficiency, the proband (III5) and other patients carry a c. 1820T > C (p.Leu607Ser) PROS1 heterozygous mutation. (b) Mutation in SERPINC1, c.883G > A (p.Val295Met). (c) The corresponding SERPINC1 wild type sequence. (d) The C.1820T > C (p.Leu607Ser) PROS1 mutation. (e) The corresponding PROS1 wild type sequence.

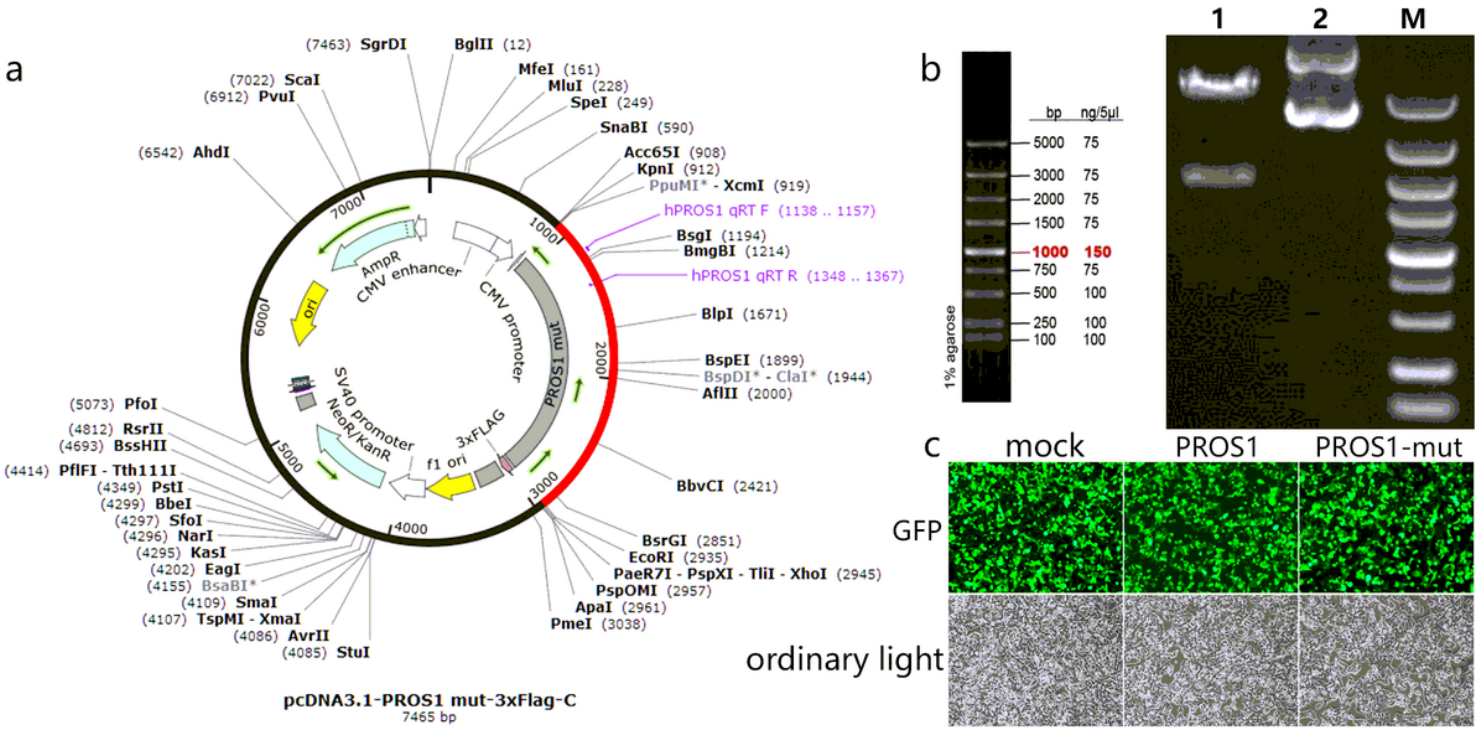


Figure 4

Protein S (PROS1) plasmid construction and verification. (a) Schematic diagram of the p.cDNA3.1-PROS1 mut-3xFlag-C plasmid vector construction. (b) PROS1 and PROS1 (L607S) plasmids have been digested with KpnI/XhoI to determine whether the constructed plasmids are correct. Lane M is the DNA Marker, Lane 1 is plasmid digested with KpnI/XhoI, and Lane 2 is plasmid DNA. (c), There is no significant difference in cell growth after transfection and green fluorescent protein (GFP) transfection efficiency among different groups.

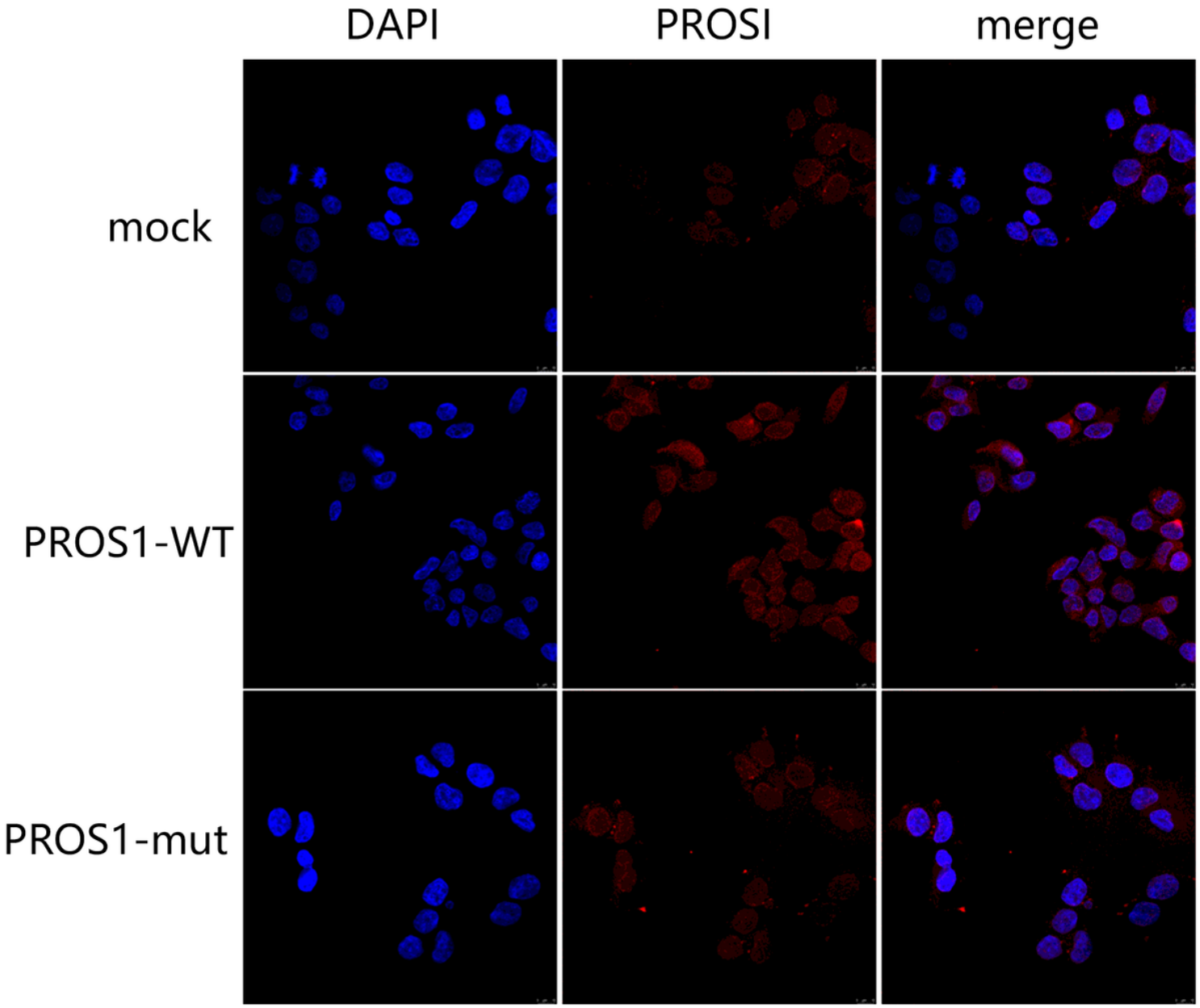


Figure 5

Localization of protein S (PROS1). Localization of PROS1 mock, wild type (PROS1-WT), and the p.Leu607Ser mutation (PROS1-M) in HEK293T cells, as detected by immunofluorescence. There is no difference before and after introduction of the PROS1 1820T > C mutation. PROS1 is mainly distributed in the cell interior.

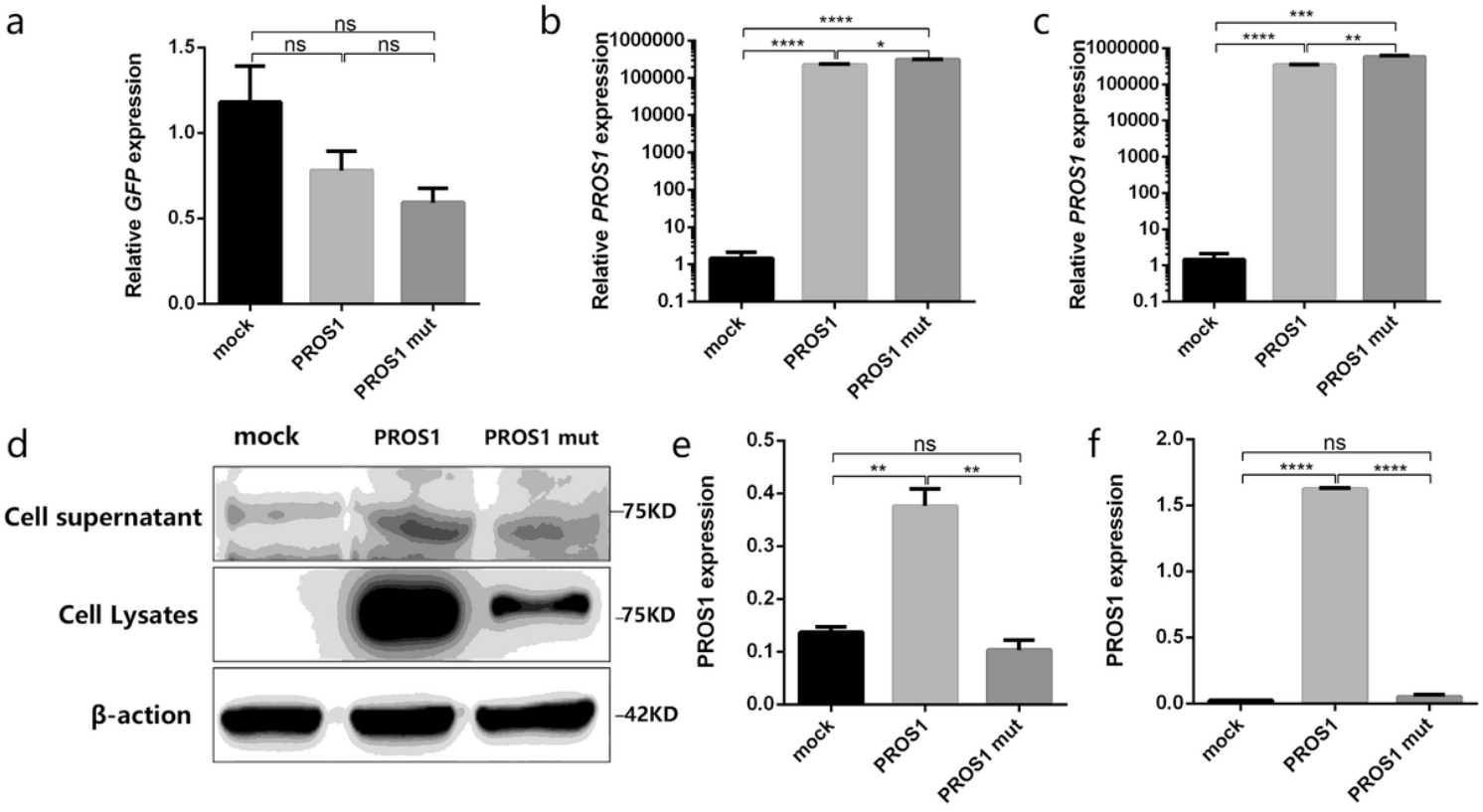
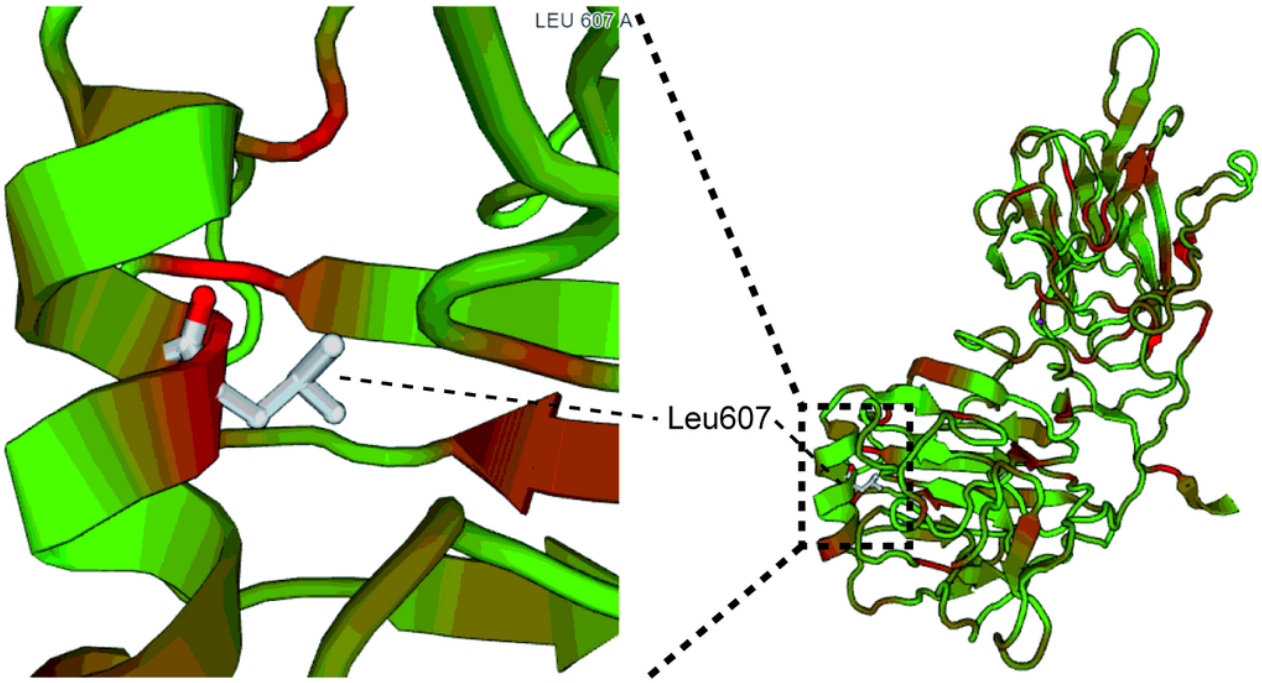


Figure 6

Relative protein S (PROS1) mRNA and protein expression. (a) Relative green fluorescent protein (GFP) expression of cells transfected with mock, wild type (PROS1-WT), and the p.Leu607Ser mutation (PROS1-M) shows that there is no significant difference in transfection efficiency between the three groups. (b) Relative PROS1 mRNA expression of mock, PROS1-WT, and PROS1-M in HEK293T cells with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. (c) Relative PROS1 mRNA expression of mock, PROS1-WT, and PROS1-M in HEK293T cells using GFP as an external reference. (d) PROS1 expression of mock, PROS1-WT, and PROS1-M in the supernatant and lysate of HEK293T cells, as detected by western blot. (e) According to the enzyme-linked immunosorbent assay (ELISA) standard curve, the expression of PROS1 mock, PROS1-WT, and PROS1-M in the supernatant of HEK293T cells is calculated. (f) According to the ELISA standard curve, the expression of PROS1 mock, PROS1-WT, and PROS1-M in HEK293T cell lysate is calculated. *p□0.05,** p□0.01,*** p□0.005, n = 3.

A



B

Homo sapiens	579	VNRNNLELSTPLKIETISHEDLQRQLAVLDKAMKAKVATYLGGLPDVPFSATPV	632
Pan troglodytes	448	VNRNNLELSTPLKIETISHEDLQRQLAVLDKAMKAKVATYLGGLPDVPFSATPV	501
Mus musculus	71	VNRNGLELWTPLRKDVIYSKDLQRQLAVLDKAMKRTVATYLGGIPDISFSATPV	124
Hyaena hyaena	578	VNRTNLELLTPLKKDIIYSEALQSQLAILDKAMKGTVATYLGGLPDIPFSATPV	631
Ochotona curzoniae	578	VNRNNLDMSTPLRKDAIHSDDLQSQLAVLDRAMRGTVTTYLGGLPDVPFNATPV	631
Myotis	614	INRTNLELVTPLKYDIIYSEDLQREFGILDKAMKGKVATYLGGLPVVPFSATPV	667
Halichoerus grypus	578	VNRNNLELFTPLKKDIXSSENLQGQLAILDKAMKGTVTTYLGGLPDIPFSATPV	631

Figure 7

Protein S (PROS1) homology modeling and analysis. (a) Homology modeling of PROS1 has been performed using Swiss-Model. Leu607 is labeled in the alpha helix. (b) Conserved analysis of amino acid sequences near Leu607 (marked with red) (https://swissmodel.expasy.org/repository/uniprot/P07225?template=1h30.1.A&range=266-673).