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ORIGINAL ARTICLE – OPEN ACCESS

Pathogenic Ala303Val Mutation in the PROS1 Gene is Associated with the Pathogenesis of Deep Vein Thrombosis

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ABSTRACT

Objective: The aim of this study was to predict the functional impact of pathogenic mutations and the mRNA expression profiles of the platelet endothelial aggregation receptor 1 (PEAR1), protein S (alpha) (PROS1), and adrenoceptor alpha 2A (ADRA2A) genes in deep vein thrombosis (DVT), as well as to examine the effects of these genes on the pathogenesis of DVT.

Materials and Methods: Patients diagnosed with DVT were selected for the study and healthy individuals were used as controls. Mutations in the PEAR1, PROS1, and ADRA2A genes were determined by DNA sequencing analysis and gene expressions were determined using quantitative real-time polymerase chain reaction testing. Polymorphism Phenotyping v2 (Polyphen-2; http://genetics.bwh.harvard.edu/pph2/), SNAP2 (https://rostlab.org/services/snap2web/) and MutationTaster (https://www.mutationtaster.org/) software were used to define the pathogenic effects of mutations detected by sequencing the selected genes in hotspot regions. Mutation and gene expression analyses were noted in the results and clinical data.

Results: A total of 27 patients with DVT and 10 healthy individuals were included in the study. Twenty-one mutations were detected in the 27 patients, most often in the PROS1 gene. A p.Ala303Val mutation is located on the human sex hormone-binding globulin (SHBG) domain of mutation PROS1 and is pathogenic. A p.A303V mutation is associated with premature termination in codon 303 of the SHBG domain. Examination of the effect on the mRNA expression level of wild-type versus mutant genotypes revealed that the mutant PROS1 p.A303V expression was significantly lower (p=0.041).

Conclusion: A p.A303V mutation in PROS1 might be an independent risk factor for DVT, which could provide helpful insight into the pathogenesis of DVT.

Keywords: ADRA2A, deep vein thrombosis, gene expression, mutation, PEAR1, platelets, polymorphism, PROS1

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INTRODUCTION

Deep vein thrombosis (DVT), one of the most common venous thromboembolic diseases, is closely linked to pulmonary embolism and post-thrombotic syndrome, both of which have a high mortality rate. DVT etiology is multifactorial, and genetic and environmental factors have synergistic effects in the formation of the disease (1–3). Risk factors for thrombosis development, whether acquired or hereditary, are relatively harmless when evaluated alone. However, the inheritance of 1 or more genetic anomalies combined with environmental hazards can make an individual a candidate for dangerous thrombosis formation (2, 3). In addition to known hereditary factors (antithrombin deficiency, Factor V Leiden [FVL], Factor II G20210A mutations), mutations/single nucleotide polymorphisms (SNPs) in genes responsible for platelet aggression can also be an independent risk factor in patients with DVT not otherwise explained, since thrombocytes are involved in the pathogenesis of DVT (2–4). In genome-wide research conducted to identify new biomarkers in recent years, several studies of candidate genes responsible for platelet aggregation have investigated the relationship between genetic changes in special genes, such as protein S (PROS1); inositol 1,4,5-triphosphate receptor associated 1 (MRVI1); Fc fragment of immunoglobulin (Ig) E, high affinity I, receptor for gamma polypeptide (FCER1G); platelet endothelial aggregation receptor 1 (PEAR1); pro-platelet basic protein (PPBP); adrenoceptor alpha 2A (ADRA2A); the sonic hedgehog (SHH) signaling molecule; and platelet aggregation (4–7). Genes that demonstrate structural and/or functional disorders may become biomarkers in the diagnosis, treatment, and prevention of recurrence of DVT, as in other types of multifactorial diseases. The present study examined 3 genes directly related to platelet aggregation: PEAR1, PROS1, and ADRA2A. The goal of this study was to predict the functional effects of pathogenic mutations and mRNA expression profiles in PEAR1, PROS1, and ADRA2A in DVT, and to clarify the effects of these genes on the pathogenesis of DVT.

MATERIALS and METHODS

The Erciyes University Faculty of Medicine Ethics Committee approved the study protocol on October 17, 2018 (project no. 2018/533) and informed consent was obtained from the participants.

Table 1. Clinico-pathological, epidemiological, and molecular data of 27 patients with DVT and healthy individuals

Characteristic	Total (n=27)	Total (n=10)	p
Gender			
Male	20 (74%)	6 (60%)	0.722
Female	7 (25.9%)	4 (40%)	0.549
Mean age, years	42 (24–70)	46 (24–70)	0.548
Site of thrombosis		–	–
Lower extremity	25 (92.5%)		
Upper extremity	1 (3.7%)		
Pulmonary embolism	1 (3.7%)		
Oral anticoagulant use		–	–
Warfarin	24 (88.8%)		
Rivaroxaban	3 (11.1%)		
Frequency of FV G/A	7/27 (26%)	1/10 (10%)	0.385
Frequency of FV A/A	6/27 (22%)	–	–
Frequency of PT G/A	4/27 (15%)	–	–
Frequency of PEAR1 mutations	10/27 (37%)	2/10 (20%)	0.468
Frequency of PROS1 mutations	27/27 (100%)	7/10 (70%)	0.525
Frequency of ADRA2A mutations	7/27 (26%)	2/10 (20%)	0.768
Hypertension	6/27 (22%)	–	–
WBC (×10^9/L)	8.22 (5.8–14.4)	8.3 (9.5–13.7)	0.998
RBC (×10^9/L)	5.22 (4.15–6.98)	4.97±1.26	0.683
aPTT	40.27±9.05	23.20 (18.90–42.20)	0.000
PTT	22.66±6.66	27±2.2	0.003
INR	1.98±0.57	0.99±1.35	0.000
Hemoglobin (g/dL)	15.01±1.58	13.2±0.8	0.358
HCT (%)	44.68±4.52	40.91±10.94	0.018
MCV (fL)	86.38±8.51	82.62±5.29	0.532
MCH (pg)	32.27±0.94	26.68±2.05	0.045
PLT (×10^9/L)	239.8 (139–355)	250.82±80.77	0.087
RDW (%)	13.86±1.11	18.59±13.25	0.132
PCT (%)	0.22±0.06	0.19±0.05	0.612
PDW (%)	16.01±0.43	14.91±1.97	0.179
MPV (fL)	9.11±1.15	8.15±1.08	0.779

The Kolmogorov-Smirnov test and the Shapiro-Wilk test were used to test the normality of the data using SPSS. The data were normally distributed. ADRA2A: Adrenoceptor alpha 2A; aPTT: Partial thromboplastin time; FV: Factor V; HCT: Hematocrit; INR: International normalized ratio; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; MPV: Mean platelet volume; PCT: Procalcitonin; PDW: Platelet distribution width; PEAR1: Platelet endothelial aggregation receptor 1; PLT: Platelet count; PROS1: Protein S (alpha); PT: Prothrombin; PTT: Thromboplastin time; RBC: Red blood cells, x10^9/L; RDW: Red blood cell distribution width; WBC: White blood cells, x10^9/L

Participants and Sample Collection

The study group consisted of 27 patients of the Nigde Omer Halisdemir University Faculty of Medicine aged 20–74 years who were diagnosed with primary DVT (n=26) and pulmonary embolism (n=1). Table 1 summarizes the clinical, genetic, and demographic data of the patients and controls included in the study.

Magnetic resonance imaging, venography, computed tomography, and color Doppler ultrasonography can be used to diagnose DVT. In the study clinic, DVT is diagnosed with venous color Doppler ultrasonography and physical examination findings. Hemogram, activated partial thromboplastin time (aPTT), prothrombin time (PT)/international normalized ratio (INR), and liver function test results are routinely used in treatment planning. Patients who had undergone surgical intervention, those who had recently been administered or were undergoing cancer treatment, those who had given birth within the previous 6 weeks, those who received hormonal therapy, those who used oral contraceptives, those with a history of acute myocardial infarction, and immobilized patients were excluded. A control group of 10 individuals (6 males, 4 fe-

males) with an age range of 20–74 years who presented at the hospital for a check-up and had no coagulation system disease or acute/chronic diseases was also enrolled.

For the DNA and RNA analysis, 2 mL blood samples were taken from the study participants in tubes containing 2% ethylenediaminetetraacetic acid.

Mutation Screening

DNA Isolation and Genotyping

The DNA isolation was performed using Zymo Quick-DNA Kits (Zymo Research Corp., Irvine, CA, USA). The isolated DNA samples were stored at -20°C for later analysis. Genomic DNA quality and concentration were determined using a spectrophotometer. The exon regions of PEAR1, PROS1, and ADRA2A to be studied were amplified using polymerase chain reaction (PCR) testing. The primers were designed using Primer3 software (https://primer3.org) (Table 2). Approximately 200 ng of genomic DNA from the amplification process was used as the template for the sequencing process. PCR was performed using a 96-well thermal cycler. Amplicons were sequenced using the CEQ 8000 genetic analysis system (Beckman Coulter Inc., Brea, CA, USA). The raw data were read using Chromas 2.6.5 software (http://technelysium.com.au/wp/chromas/) to detect mutations defined according to reference sequences obtained from the Ensembl genome browser (https://www.ensembl.org). Genotyping of FVL and Factor II mutations were screened using real-time PCR (RT PCR) melting curve analysis of fluorescence with a LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). The primers and probes used in the study are summarized in Table 2. Melting analysis was performed using LightMix *in-vitro* diagnostics FVL and Factor II G20210A mutation detection kits (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

In-silico Analysis of the PROS1, PEAR1, and ADRA2A Pathway Mutations

Polymorphism Phenotyping v2 (Polyphen-2; http://genetics.bwh.harvard.edu/pph2/) software helps to determine effects on the stability and function of the protein in question using physical and comparative data of amino acids affected by mutations in genes. The resulting estimation is based on a number of phylogenetic, sequence, and structure-based properties characterizing the amino acid changes. The program provides a qualitative prediction of probably damaging, possibly damaging, benign, or unknown (8). The evolutionary conservation analysis of the mutant codons identified during the study with the multiple sequence alignment tool included in this software was compared between species.

SNAP2 (https://rostlab.org/services/snap2web/) software is a tool that scores the impact of variants/non-synonym single nucleotide polymorphisms (SNPs) by considering various sequence and variant properties. Values between -100 and 0 are scored as neutral, while a value of 0 to 100 is scored as having an effect (9). Finally, MutationTaster (https://www.mutationtaster.org/), which uses a Bayes classifier, was used to predict the disease potential of a variant (10). Structural

Table 2. The primer sequences and amplicon lengths for PEAR1, PROS1 and ADRA2A genes

Gene/exon	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon(bp)
PEAR1 (intron-1)	CTAAGTTCTCCCCCTGAGTT	TAGGGAGGGTGTACCTGAG	339
PEAR2 (exon-12)	TGAGGTCTGAGGTTCTGTTC	GGTTGACAAGGCAGAATG	331
PROS1 (exon 3-1)	GAGTTTGTGTTTAGGACGAAAA	TTCTTCTGCCCTTATCTGCT	469
ADRA2A (exon-1)	CTCTGCGTTTCCTCGTCT	TATATTTACAGCGGGGGATG	382
PROS1 (m-RNA)	TCCTGGTTAGGAAGCGTCGT	CCGTTTCCGGGTCATTTTCAAA	132
ADRA2 (m-RNA)	AGAAGTGGTACGTCATCTCGT	CGCTTGGCGATCTGGTAGA	96
FV G1691A	TGCCCAG TGCTTAACAAGACCA	CTTGAAGGAAATG CCCCATTA	110
PT G20210A	CCGCTGGTATCAAATGGGG	CCAGTAGTATTACTGGCTCTTCCTG	106
FVG1691A Hybridization probes	5'-LC-Red705 TGTCCTTGAAGTAACCTTTCAGAAATTCTG-3'-PHO	5'GGCGAGGAATACAGGTAT-3'-Flu	N/A
PTG20210A Hybridization probes	5'-LC-Red640-TCCCAGTGCTATTCATGGGC-3'-PHO	5'-CTCAGCGAGCCTCAAT3'-Flu	N/A

Genotyping polymerase chain reaction (PCR) testing: The total volume of the reaction mix was 25 µL, comprising 16.75 µL sterile water, 2.5 µL reaction buffer (200 mM (NH4)2SO4), 1 µL dNTP mix (A, C, G, T; 200 mM), 1.25 µL MgCl2 (25 mM), 1 µL forward and reverse primers (10 pmol), 0.5 µL Taq polymerase (500 units; Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 1 µL template DNA (>500 ng/µL for each sample). Amplification reactions were performed using a Veriti VR 96-well thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Thermocycling conditions were as follows: Initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation for 45 s at 94 °C, primer annealing for 90 s at 58 °C, extension for 90 s at 72 °C, and final extension for 10 min at 72 °C. Following amplification, 10 µL PCR products were separated using electrophoresis and a 2% agarose gel containing GelRed in 1X TAE buffer (Tris base, acetic acid and EDTA) for 40 min at 90 V. A GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as a marker. PCR testing was performed using the Applied Biosystems 96-well thermal cycler (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Thermocycling conditions were as follows: Initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation for 45 s at 94 °C, primer annealing for 90 s at 58 °C, extension for 90 s at 72 °C, and final extension for 10 min at 72 °C. ADRA2A: Adrenoceptor alpha 2A; PEAR1: Platelet endothelial aggregation receptor 1; PROS1: Protein S (alpha)

Table 3. Characteristic and clinical features of PROS1, PEAR1 and ADRA2A gene mutations in patients with DVT

No.	Gene	Nt alteration	Rs No.	Alteration type	Localization	AA position	Number of patients carrying changes	Previously determined disease/ browser	Clinical significance		
									Poly-Phen2	SNAP	Mutation taster
C-1	PROS1	c.886A>C	rs1559932774 /COSV67776681	Missense mutation	Exon-9/ SHBG domain splice site changes	p.K296Q	P3,P5,P7,P12, P21,P23, P26,P27	Liver tumor	0.148 (Benign)	49 (Effect)	Polymorphism protein may be affected
C-2	PROS1	c.919G>T	rs761691331	Missense mutation	Exon-9/ SHBG domain	p.A307S	P19,P20,P21	GNOMAD	0.44 (Benign)	-84 (Neutral)	Polymorphism known disease mutation at this position (HGMD CI972678)
C-3	PROS1	c.947G>A	COSV67776384/ rs747259055	Missense mutation	Exon-9/ SHBG domain splice site changes	p.R316H	P4,P5,P6, P21,P23	Skin/liver tumor	0.007 (Benign)	-27 (Neutral)	Polymorphism protein may be affected
C-4	PROS1	c.909G>A	COSV6777741 rs537938359	Silent mutation	Exon-9/ SHBG domain splice site changes	p.A303=	P1,P15,P16,P17,P18, P19,P20,P21,P22,P23, P24,P25,P26,P27	1000 Genome Project	NA	NA	Disease causing
C-5	PROS1	c.908 C>T	COSV67776359	Missense mutation	Exon-9/ SHBG domain splice site changes	p.A303V	P1, P2,P7,P9, P12, P21,P23,P25,P26, P27	Lung tumor	0.98 (Probably damaging)	45 (Effect)	Disease causing

ADRA2A: Adrenoceptor alpha 2A; C: Change; DVT: Deep vein thrombosis; NA: Not available; P: Patient; PEAR1: Platelet endothelial aggregation receptor 1; PROS1: Protein S (alpha) ; SHBG: Sex hormone-binding globulin; SNP: Single nucleotide polymorphism; UNK: Unknown; UTR: Untranslated region

modeling of selected mutations was calculated using the SWISS-MODEL server (https://swiss-model.expasy.org), stand-alone software that can generate mutated models of the proteins for the corresponding amino acid substitutions (11). The Catalog of Somatic Mutations in Cancer (COSMIC) database (https://cancer.sanger.ac.uk) was used to determine the possible pathogenicity of the identified mutations (12).

PROS1 and ADRA2A mRNA Expression Analysis

RNA Isolation, c-DNA Synthesis, and Quantitative Real-Time PCR

RNA isolation was performed using PureZOL solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's recommended protocol. A spectrophotometer was used to defined the concentration and purity of the samples of RNA, which were then stored at -80°C until use. The primer sequences are given in Table 2. In this study, the quantitative RT PCR (q-RT PCR) technique was performed to calculate the mRNA expression rates of the human acidic ribosomal protein (RPLP0) housekeeping gene in PROS1 and ADRA2A. The manufacturer's recommended protocol for use of the c-DNA synthesis kit was followed. The mRNA levels of the study group were determined according to the protocol proposed by the manufacturer using SYBR Green I Master (Thermo Fisher Scientific, Waltham, MA, USA) and mRNA levels were established with Rotor-Gene Q (Qiagen, Hilden, Germany). Each sample was studied in 3 replicates. The mRNA levels were measured according to 2exp-ΔCt (13).

Statistical Analysis Power analysis was performed to determine the number of samples to be included in the study; α=0.05 and 1-β=0.80 yielded a sample size of 100. The statistical power was 0.80. A Cohen's d moderate effect size of 0.25 was determined, using the information obtained from the literature as a case-control study. The normality of the distribution of the data in the groups was confirmed using the Kolmogorov–Smirnov test. Analysis of variance (ANOVA) and chi-squared testing were used as appropriate to compare clinico-pathological, epidemiological, and molecular data. The calculation of amplification efficiency was an important step in the qRT-PCR analysis of RNA used to calculate comparative expression. Statistical analysis of mRNA analysis was performed using the one-way ANOVA feature of IBM SPSS Statistics for Windows, Version 22.0 software (IBM Corp., Armonk, NY, USA). A p value of <0.05 was considered statistically significant.

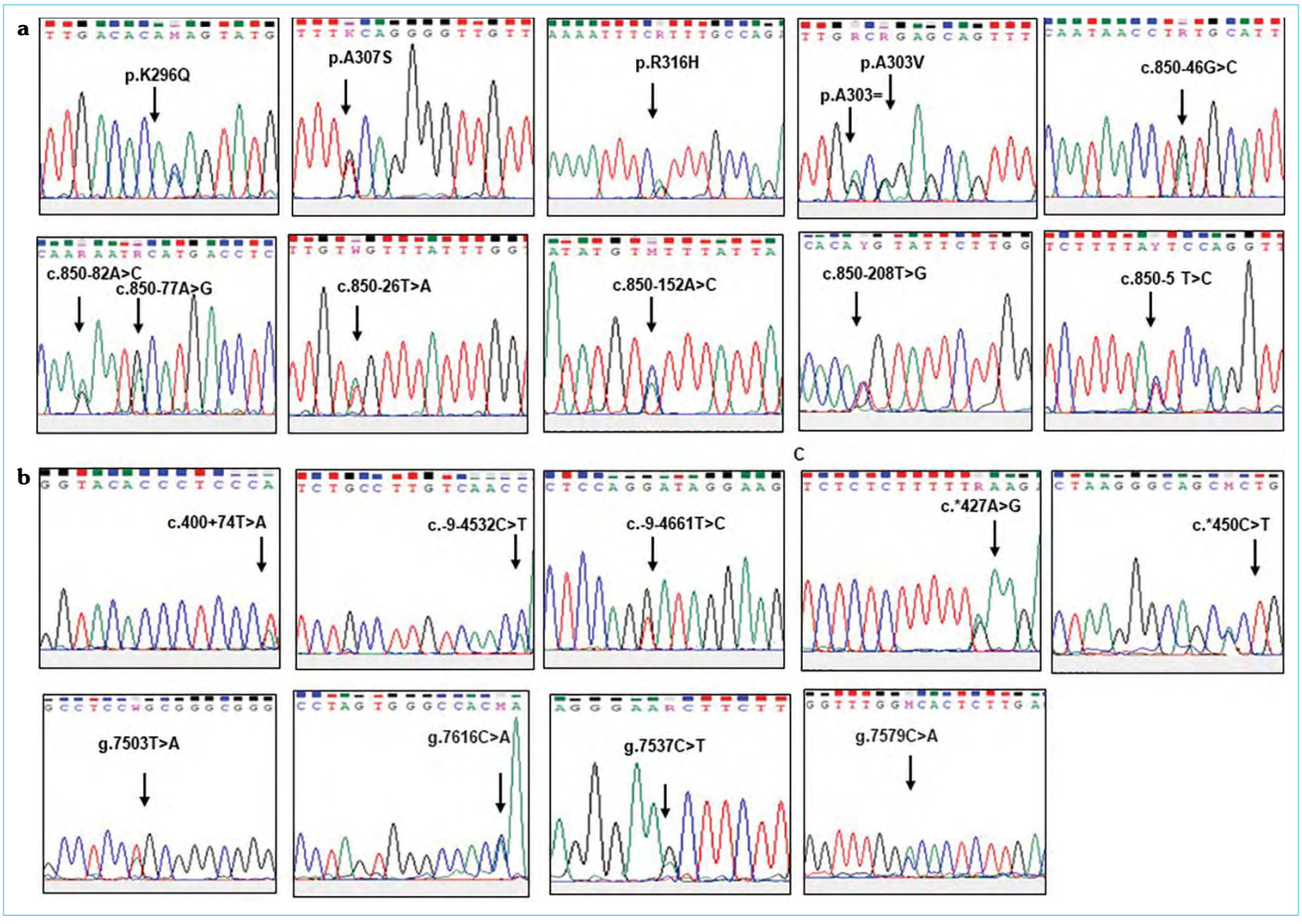


Figure 1. Sequencing electropherograms of (a) PEAR1, (b) PROS1, and (c) ADRA2A gene mutations and variants. Arrows indicate the localization of the mutations on the domains

ADRA2A: Adrenoceptor alpha 2A; PEAR1: Platelet endothelial aggregation receptor 1; PROS1: Protein S (alpha)

RESULTS

Clinical and Demographic Analysis of DVT Patient Group

The mean age of the patient group was 42 years (range: 20–74 years). Examination of the gender distribution indicated that 20 (74%) of the 27 patients were male and 7 (26%) were female. DVT was determined in the upper extremity veins in 1 patient (P27), in the pulmonary artery in 1 patient (P12), and in the lower extremity veins in 25 members of the patient group. Eight patients had a history of hypertension (P7, P9, P12, P16, P20, P21, P22, and P24). Warfarin was used in the treatment of 24 patients, and rivaroxaban was used in 3 patients (P3, P4, and P6). Table 1 provides the demographic, clinical, and diagnostic parameters of the study group.

Results of Mutation Screening

The Sanger method of DNA sequencing was used to detect mutations. In all, 21 (4 missense mutations, 1 silent mutation, 16 SNP) changes were found. Nineteen were previously registered in the Human Gene Mutation Database (Cardiff University, Cardiff, Wales), while 2 were defined for the first time in this study. The detected mutations had a heterozygous genotype. Changes in the PROS1, PEAR1, and ADRA2A genes were detected in 26 of 27 DVT patients (no change in P8), which are illustrated in the electropherograms in Figure 1. The genetic risk factors associated with thrombosis in our study group were FVL and PT mutations, with a frequency of 13/27 (48%) and 4/27 (15%), respectively. None of the healthy control subjects had an identified mutation of PT, but a heterozygous mutation of FVL was detected in 1 (10%) participant. Table 3 provides details of the changes detected in 3 genes in the DNA sequencing analysis. The schematic presentation of the genes and mutations observed is presented in Figure 2. The number of patients and controls with PEAR1, PROS1, or ADRA2A mutations and the risk caused by the alterations is shown in Table 4.

PROS1 Analysis

The greatest number of changes was found in PROS1 (12 changes). Four of the 12 were missense mutations: p.Lys296Glu (p.K296Q) was identified in 8 (30%) of 27 patients, p.Ala307Ser (p.A307S) in 3 (11%) patients, p.Arg316His (p.R316H) in 5 (18.5%) patients, and p.A303V in 9 (33%) patients. The p.K296Q, p.R316H, p.Ala303Ala (p.A303=) and p.Ala303Val (p.A303V) mutations have been recorded as somatic mutations in the COSMIC database with reference numbers COSV67776681, COSV67776384, COSV6777741, and COSV67776359, respectively. The p.A303V mutation has been noted as a risk factor

Table 4. Combined risk effect of PROS1 mutations on DVT patients with and without FVL mutation

Mutations	DVT n=27 (%)	FVL$^+$ mutation n=13(%)	FVL$^-$ mutation n=14(%)	OR	CI (95%)
PROS1/p.K296Q	8 (29.6)	5(38.4)	3 (21.4)	0.55	0.11–2.57
PROS1/p.A307S	3 (11.1)	2(15.3)	1 (7.1)	0.48	0.04–5.6
PROS1/p.R316H	5 (18.5)	3 (23)	2 (14.2)	0.64	0.09–4.17
PROS1/p.A303=	14 (51.8)	6 (46.1)	8 (57.1)	1.47	0.43–5.02
PROS1/p.A303V	10 (37)	4 (30.7)	6 (42.8)	1.64	0.40–6.63

CI: Confidence interval; OR: Odd ratios; DVT: Deep vein thrombosis; FVL: Factor V Leiden; PROS1: Protein S (alpha)

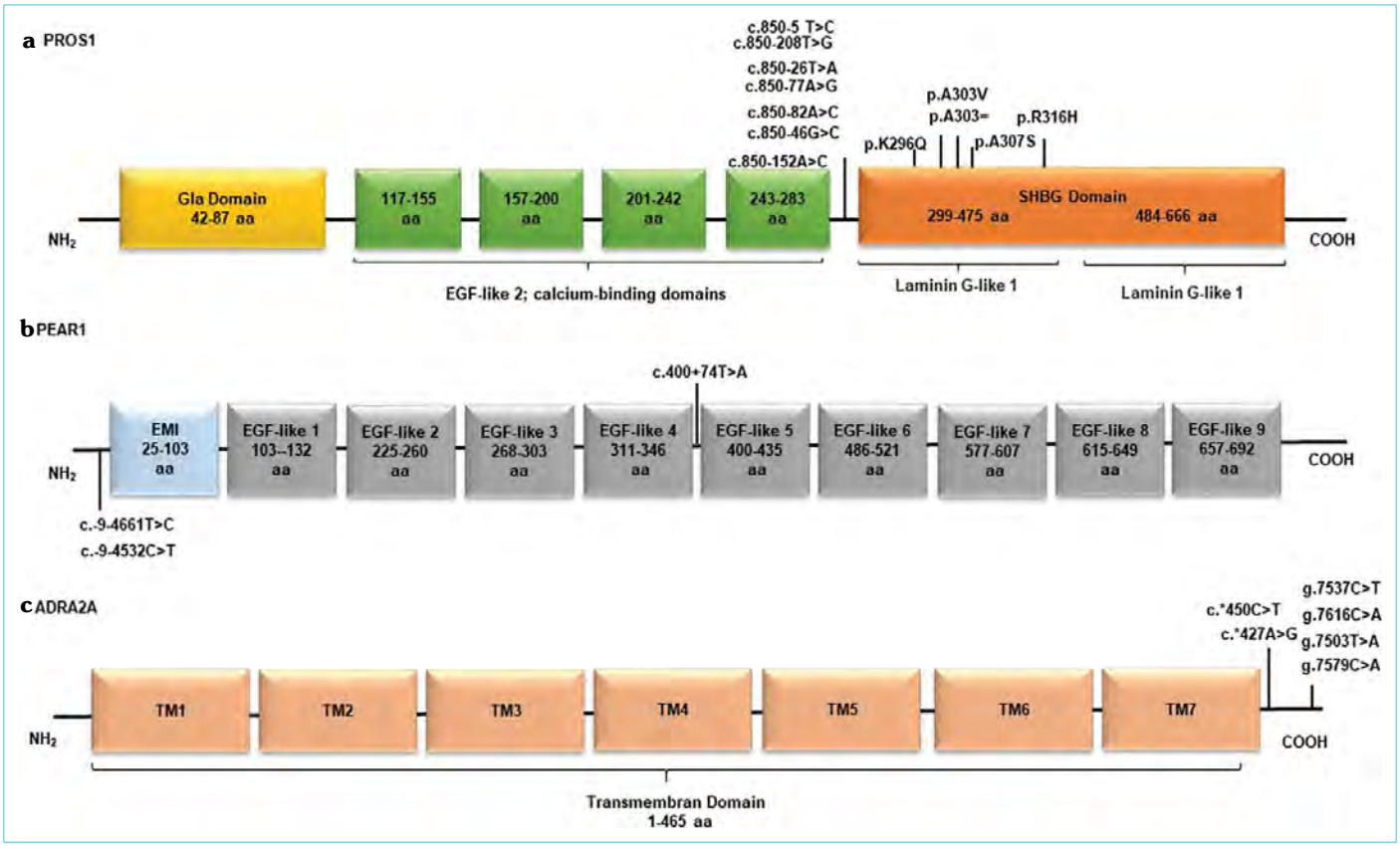


Figure 2. Schematic representation of the domain structure of the PEAR1, PROS1, and ADRA2A proteins and mutations detected in patients with deep vein thrombosis. (a) PROS1 is a polypeptide of 676 amino acids; (b) PEAR1 is a polypeptide of 1037 amino acids; (c) ADRA2A is a polypeptide of 465 amino acids

for lung adenocarcinoma. p.K296Q, p.R316H, p.A303=, and p.A303V changes are splice-site mutations. The p.A303V mutation is located in the laminin G-like 1 (LG-1)/SHBG-like domain, which has a key role in the expression of the active protein C cofactor anticoagulant (14).

PEAR1 Analysis

A total of 3 changes were detected in the PEAR1 gene, all of which were in the non-coding region.

ADRA2A Analysis

Six changes were detected in the ADRA2A gene. The c.*427A>G change was in the 3' untranslated region (UTR). This alteration may be in the miRNA binding region and may be responsible for mRNA stabilization.

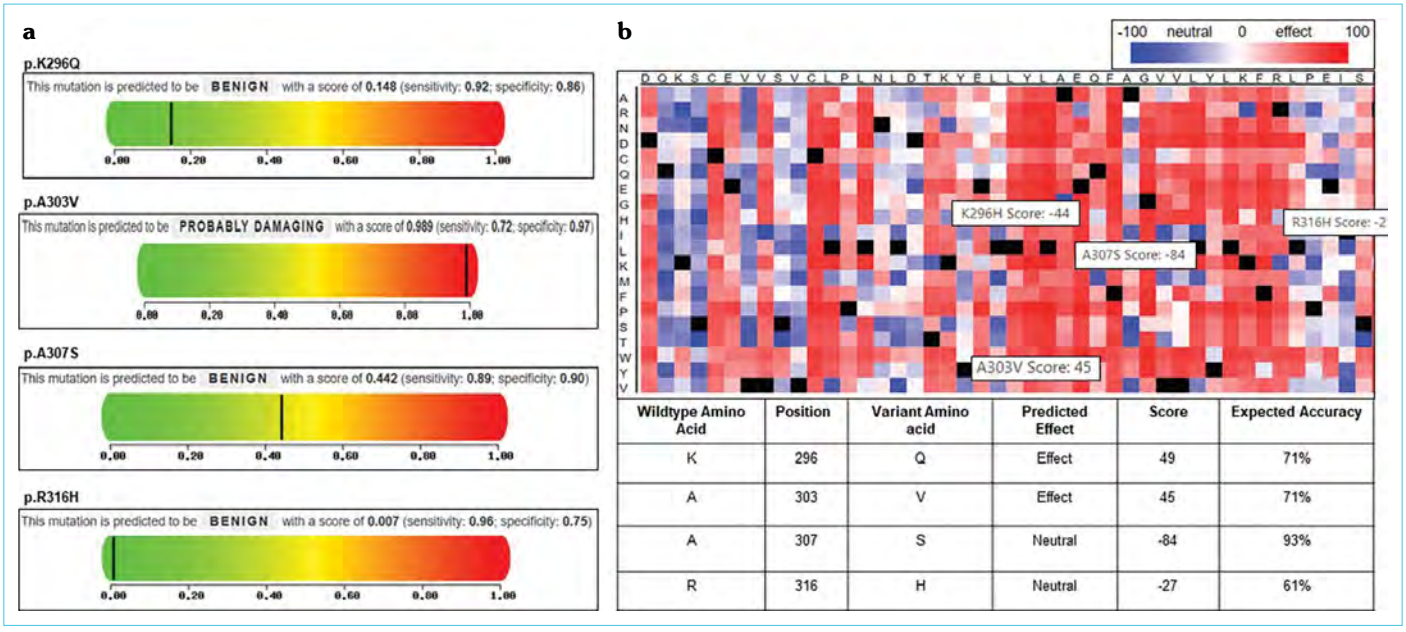
Results of In-silico Analysis

Pathogenicity Prediction Analysis

The Poly-Phen2, SNAP2, and MutationTaster analyses indicated that the mutations detected in our study could be pathogenic, especially since the PROS1 p.A303V pathogenic score was close to 1, which signifies an effect. MutationTaster reported p.A303V as "disease causing". Figure 3 (a–d) provides the detailed predictive pathogenic analyses conducted with Poly-Phen2 and SNAP2.

Structure Modeling Analysis

The SWISS-MODEL Interactive Workspace (https://swissmodel.expasy.org/interactive) was used to model the molecular structure of wild-type and mutant proteins. Figure 4 (a–f) shows the location of affected PROS1 codons and the molecular structures of wild-type and mutant proteins. The p.A307V mutation caused a significant conformational change.



Wildtype Amino Acid	Position	Variant Amino acid	Predicted Effect	Score	Expected Accuracy
K	296	Q	Effect	49	71%
A	303	V	Effect	45	71%
A	307	S	Neutral	-84	93%
R	316	H	Neutral	-27	61%

Figure 3. Estimation of possible functional effects of mutations in the PROS1 IL6 gene using (a) PolyPhen-2 (a) and (b) SNAP2 software

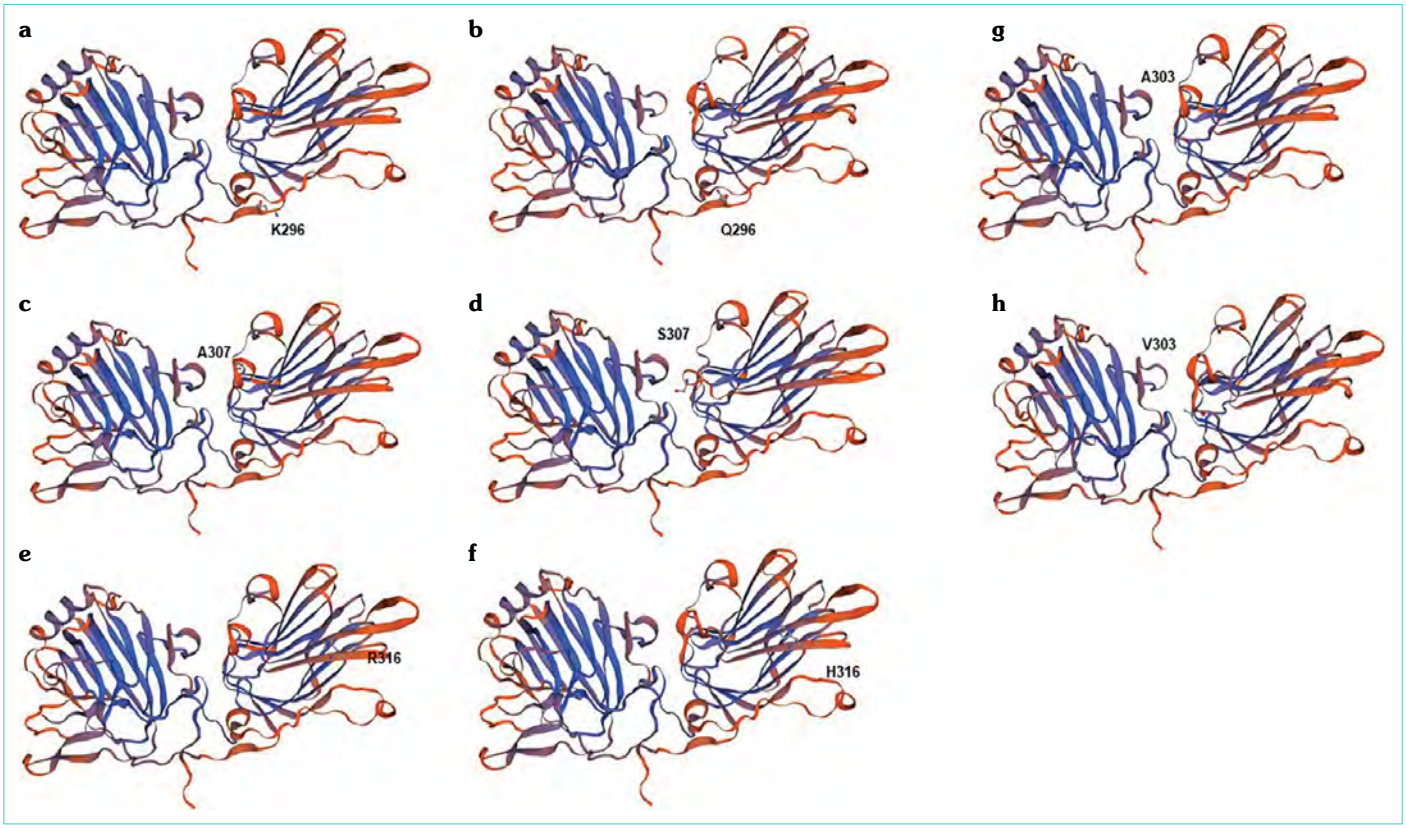


Figure 4. Structure models of wild-type and mutant protein S. (a, b) Protein S wild-type and K296Q mutation; (c, d) Protein S wild-type and A307S mutation; (e, f). Protein S wild-type and R316H mutation; (g, h). Protein S wild-type and A303V mutation

Evolutionary Conservation Analysis

Multiple sequence alignment analysis of the amino acid codons affected by the variants was used to compare several species for the effect of missense mutations. PROS1 p.A303V, p.A307V, and p.R316H mutations were found to change the amino acids located at a critical point during the evolutionary process. The analysis of preservation in the evolutionary process is presented in Figure 5 (a–d).

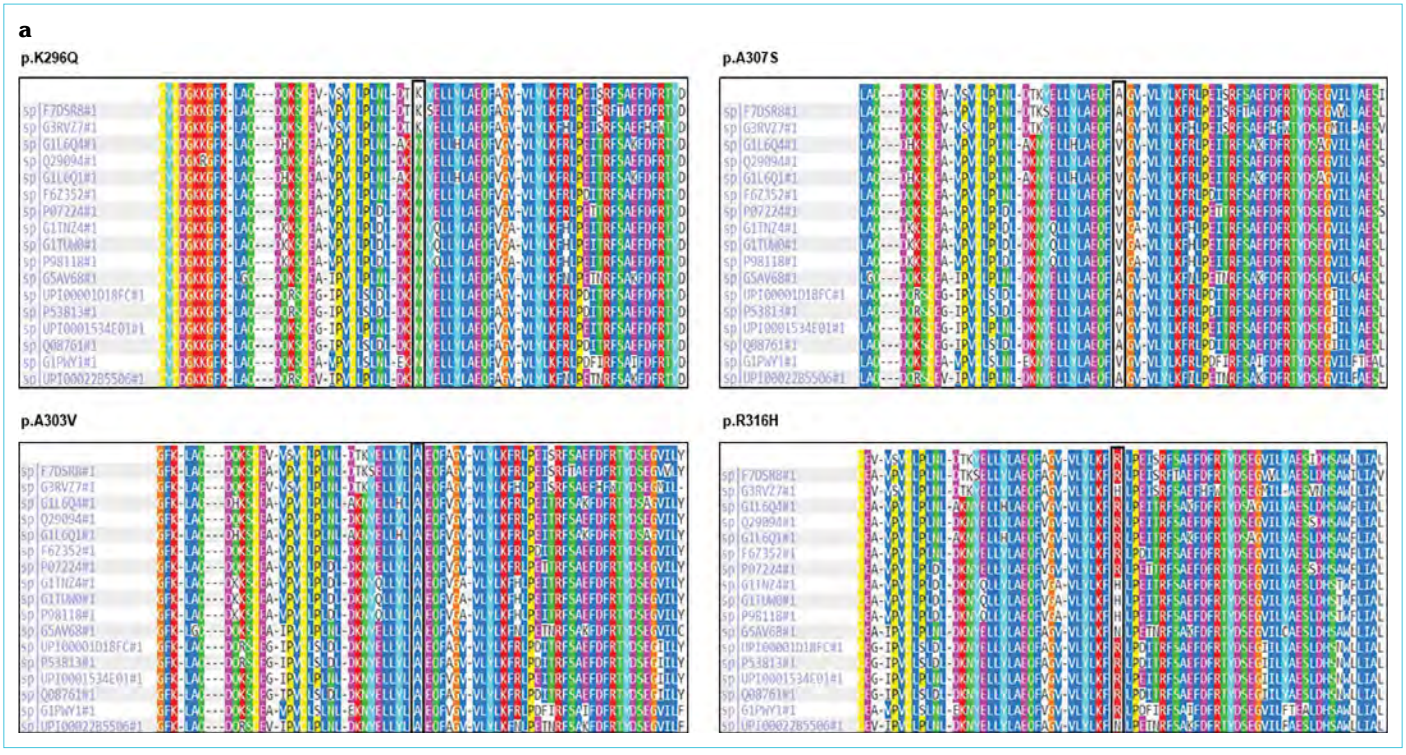


Figure 5. Evolutionary conservation analyses of amino acid mutations in the Protein S (alpha) (PROS1) gene in different species

Results of PROS1 and ADRA2A mRNA Expression Analysis

Simultaneous qRT-PCR was used to examine the mRNA expression rate in the PROS1, ADRA2A, and housekeeping genes in 27 patients and 10 controls. As shown in Figure 6a, the mRNA expression rate of PROS1 was higher than that of ADRA2A, but without statistical significance in our DVT patients, as revealed by the mRNA levels normalized to the control group (p=0.295). Patients were divided into mutant and wild-type groups in the analysis to determine the effect of a PROS1 p.A303V mutation on mRNA level. Comparison of the mRNA levels of these groups indicated that the level of changes in PROS1 was significant (p=0.041) (Fig. 6 b, c) The changes detected in PEAR1 were not included in the qRT-PCR analysis as they were not in the non-coding region of the gene nor in the 3'UTR that enables mRNA stability.

DISCUSSION

The main causes of DVT include venous stasis, endothelial damage, and hypercoagulability. In addition, advanced age, obesity, pregnancy and puerperium, some tumor diseases, orthopedic surgery, prolonged bed rest, sitting still for long periods (for instance during travel), certain medications (estrogen-containing oral contraceptives, hormone therapy during menopause, etc.), innate coagulation disorders that occur in the later stages of life, and genetic factors all increase the risk of DVT (1–4, 15). Many patients with >1 risk factor may never develop DVT, yet conversely, cases of DVT often cannot be associated with any known clinical risk factor. It is crucial to establish new hypotheses that can provide a better understanding of the normal biology of human deep veins and make clear the pathogenesis of DVT.

The objective of present study was to examine the influence of PROS1, PEAR1, and ADRA2A, which are involved in platelet aggregation, on the pathogenesis of DVT. To the best of our knowledge, our study is the first in the literature to assess the mutation and gene expression profile of PROS1, PEAR1, and ADRA2A in DVT patients and provide a comprehensive analysis. First, genotyping of regions encoding important domains of the studied genes was performed in 27 patients with DVT using DNA sequencing. A total of 21 changes in PEAR1, PROS1, and ADRA2A were detected: 4 missense mutations, 1 silent mutation, and 16 SNPs. Our study was the first to describe 2 of these changes (PROS c.850-5 T>C; PEAR1 c.400+74T>A). The greatest number of changes was observed in PROS1 (57%). Protein S (PS) deficiency is an independently-acquired inherited risk factor for DVT. The incidence of PS deficiency in venous thrombosis has been reported to be 2% to 15% (16–18). Studies have reported that genetic changes in PROS1 have a negative effect on the functionality of PS. Some 360 genetic mutations linked to PS deficiency have been recorded in the mutation database thus far (20, 19–21). The 4 missense mutations detected in our study were in PROS1 (p.K296Q, p.A307H, p.R316H, p.A303V). The detected mutations are in the SHBG domain of the gene. This SHBG domain (amino acid 392) consists of sequential repeats of a domain first identified in the region of the laminin A chain and is therefore referred to as an LG-domain. It carries residues (amino acids 243–635) containing 2 (LG) domains (LG1/LG2) that mediate interactions with C4b-binding protein (14, 21, 22). According to the results of in-silico data analysis, the p.A303V mutation may be pathogenic: the pathogenic score was close to 1 and it was graded "effect", "probably damaging", and "disease causing". The p.Leu259Pro (p.L259P), p.Ser283Pro (p.S283P), p.Leu310Pro (p.L310P), p.Leu405Pro (p.L405P), p.Arg474Pro

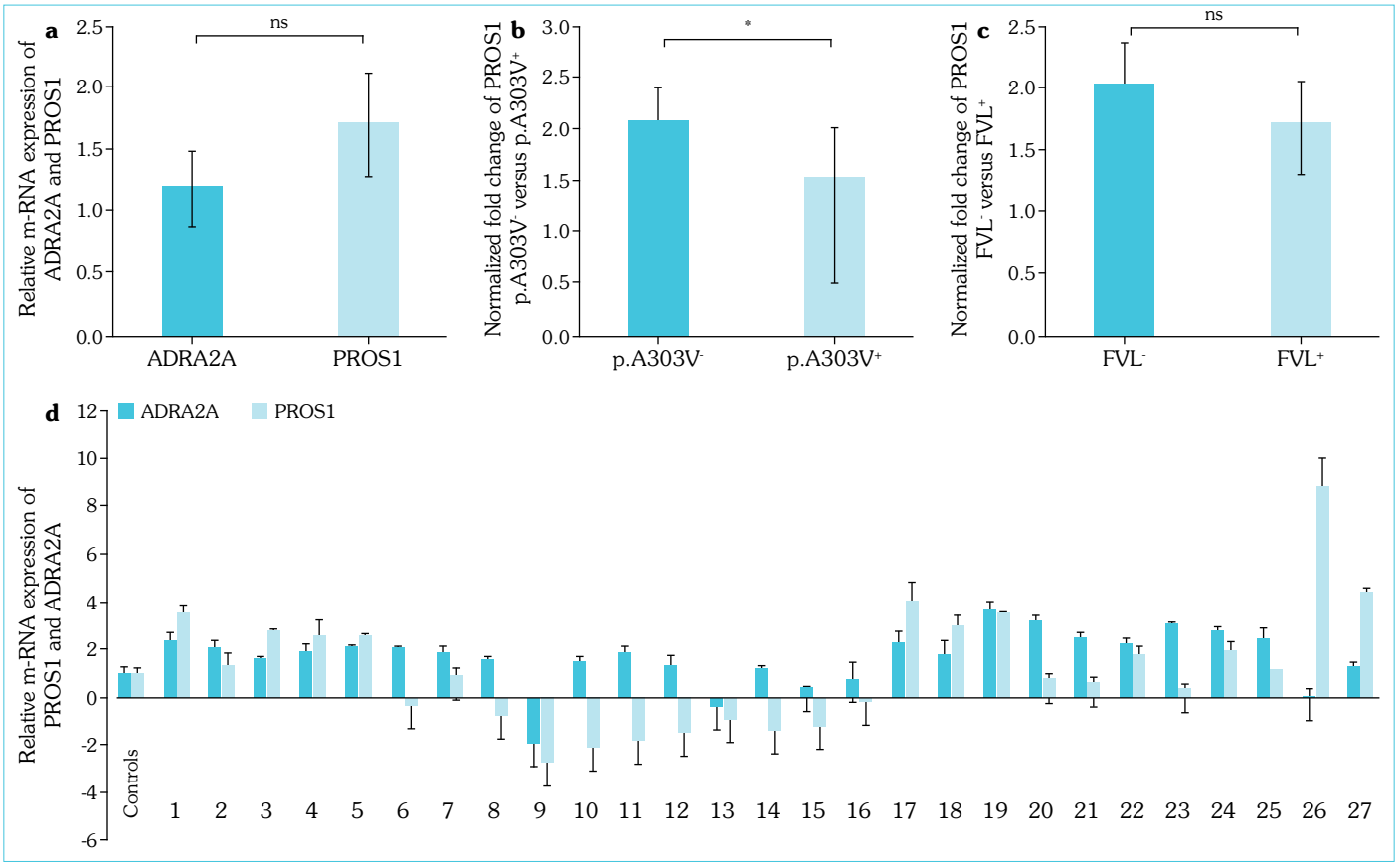


Figure 6. (a) Normalized relative gene expression levels of PROS1 and ADRA2A in patients with deep vein thrombosis; (b, c). Comparative analysis of mRNA expression value of mutant and wild-type individuals in PROS1 and FVL genes; (d) Comparison of PROS1/ADRA2A gene expression levels according to patients

*: Indicates p<0.05; NS: Non-significant

(p.R474P), p.Ala575Pro (p.A575P), and p.Ala484Pro (p.A484P) mutations have been reported in previous studies to cause disruption of the secondary structure (14, 16). The p.A303V mutation detected in our study is in the early part of the LG1 domain. We have determined that it has been preserved among species throughout the evolutionary process, and we argue that it may disrupt the accurate folding properties of the protein that will be formed due to its effect on conserved critical amino acids. Since PS has anticoagulant properties independent of activated protein C as a result of directly inhibiting prothrombinase complexes, a p.A303V mutation may disrupt the functioning of PS. A p.A303V mutation was identified in 1 patient (P7) with a FVL homozygous genotype and 3 patients (P21, P25, and P27) with a heterozygous genotype. The p.K296Q, p.R316H p.A303=, and p.A303V splice region mutations detected in PROS1 were determined as a result of in-silico analysis. We presume that splicing activity may prevent the formation of a functional transcript due to its disruptive nature, however, these results must be verified by additional experimental studies.

The study patients were also classified into mutant (A303V+) and wild-type (A303V-) groups to determine the effect of the PROS1 A303V mutation on mRNA levels. Comparison revealed that individuals with p.A303V+ had substantially lower expression levels (p=0.041). A PS deficiency is a known independent risk factor for DVT. The majority of identified PS deficiencies caused by PROS1 mutations have been established to be due to missense or nonsense mutations in the gene's coding regions or regulatory sequences. Individuals who carry both the FVL mutation and the A303V mutation, another known thrombosis risk factor, have an increased risk of thrombosis.

PEAR1 plays a vital role in hemostasis as a platelet receptor. Extracellular matrix adhesion prompts platelet aggregation (22, 23). Some polymorphisms in the gene are known to increase thrombocyte aggregation and impair platelet function (4, 7, 23). PEAR1 is a receptor tyrosine kinase that mediates platelet-platelet adhesion through the EMI domain (EMILIN family) in its extracellular components and triggers PI3K/AKT/PTEN signaling, leading to PEAR1-mediated megakaryopoiesis and neoangiogenesis stimulation (4, 22–24). Three SNPs detected in PEAR1 are on the non-coding region. PEAR1 fulfills its platelet function by controlling the PTEN and PI3K pathways (23). Studies conducted with African-American patients and Americans of European descent have determined that intronic variants in PEAR1 had a strong association with platelet aggregation (22–26). ADRA2A, a member of the G protein-coupled receptor superfamily, is a type of adrenergic receptor that inhibits adenylate cyclase (4, 27–29). ADRA2A on platelets interacts with epinephrine, which plays a vital role in the regulation of platelet function. There is familial clustering of inter-individual variations in epinephrine-induced platelet aggregation, but the molecular mechanism is still poorly understood. (28, 29).

A total of 6 SNPs were detected in ADRA2A, the final gene examined in our study. c.*427A>G (rs553668) is in the 3'UTR section of the gene, which is a miRNA binding region, and has been identified as a risk factor in type 2 diabetes. Researchers have detected 4 SNPs in ADRA2A (rs1800545, rs4311994, rs11195419, and rs553668) in DVT patients, which indicates that ADRA2A is associated with thrombocyte hyperaggregability. This, in turn, may manifest as venous thromboembolism.

Gene expression analysis using the using qRT-PCR technique was applied to determine the effect of mutations in the mRNA expression rates of PROS1 and ADRA2A in 27 patients and 10 healthy individuals. The PROS1 expression rate was higher than that of ADRA2A, but the difference was not statistically significant (p=0.295).

It has been established that the risk of developing a thrombotic complication is about 5–10 times greater in individuals with heterozygous FVL than in the healthy population and 80–100 times greater than homozygotes. The incidence of an FVL mutation in our country's healthy population has been reported to be about 7.9% (30). The incidence was 48% in our DVT patient group. This suggests that our study group was a well-selected primary DVT patient group with no other underlying causes and would appear to confirm that FVL mutations are a main actor in the pathogenesis of DVT.

CONCLUSION

In summary, 21 changes (4 missense mutations, 1 silent mutation, 16 SNPs) were detected in this analysis of the PROS1, PEAR1, and ADRA2A genes, 2 of which were identified for the first time in the current research. We argue that a p.A303V PROS1 mutation may be an independent risk factor for DVT due to its pathogenicity. However, the number of samples involved in the study was limited, so the target genes should be analyzed in a larger study group to draw a definitive conclusion about their role in the pathogenesis of DVT and to determine biomarker potential. Therefore, our data must be validated with larger samples in order to be used for diagnostic purposes. Our study data represent preliminary data for future studies. Nonetheless, this study is important in terms of understanding the frequency and molecular features of the signal pathway mutations detected in DVT.

Ethics Committee Approval: The Erciyes University Clinical Research Ethics Committee granted approval for this study (date: 17.10.2018, number: 2018/533).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – DFAB; Design – TE, DFAB; Supervision – DFAB, DTO, TE; Resource – DFAB; Materials – TE; Data Collection and/or Processing – DFAB, TE; Analysis and/or Interpretation – DFAB, DTO; Literature Search – DFAB, DTO; Writing – DFAB, DTO; Critical Reviews – DFAB, DTO, TE.

Conflict of Interest: The authors have no conflict of interest to declare.

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REFERENCES

1. Bafunno V, Margaglione M. Genetic basis of thrombosis. Clin Chem Lab Med 2010; 48 Suppl 1: S41–51. [CrossRef]
2. Di Nisio M, van Es N, Büller HR. Deep vein thrombosis and pulmonary embolism. Lancet 2016; 388(10063): 3060–73. [CrossRef]
3. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. N Engl J Med 2001; 344(16): 1222–31. [CrossRef]
4. Sokol J, Skerenova M, Ivankova J, Simurda T, Stasko J. Association of genetic variability in selected genes in patients with deep vein thrombosis and platelet hyperaggregability. Clin Appl Thromb Hemost 2018; 24(7): 1027–32. [CrossRef]
5. Shaydakov ME, Diaz JA, Comerota AJ, Lurie F. Targeted gene expression analysis of human deep veins. J Vasc Surg Venous Lymphat Disord 2021; 9(3): 770–80.e7. [CrossRef]
6. van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. Nat Rev Cardiol 2019; 16(3): 166–79. [CrossRef]
7. Qayyum R, Becker LC, Becker DM, Faraday N, Yanek LR, Leal SM, et al. Genome-wide association study of platelet aggregation in African Americans. BMC Genet 2015; 16: 58. [CrossRef]
8. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013; Ch 7: Unit7.20. [CrossRef]
9. Bromberg Y, Rost B. SNAP: predict effect of non-synonymous polymorphisms on function. Nucleic Acids Res 2007; 35(11): 3823–35.
10. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods 2014; 11(4): 361–2. [CrossRef]
11. Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T. The SWISS-MODEL Repository and associated resources. Nucleic Acids Res 2009; 37(Database issue): D387–92. [CrossRef]
12. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, et al. COSMIC: The catalogue of somatic mutations in cancer. Nucleic Acids Res 2019; 47(D1): D941–7. [CrossRef]
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402–8. [CrossRef]
14. Villoutreix BO, Dahlbäck B, Borgel D, Gandrille S, Muller YA. Three-dimensional model of the SHBG-like region of anticoagulant protein S: new structure-function insights. Proteins 2001; 43(2): 203–16. [CrossRef]
15. Kruger PC, Eikelboom JW, Douketis JD, Hankey GJ. Deep vein thrombosis: update on diagnosis and management. Med J Aust 2019; 210(11): 516–24. [CrossRef]
16. Do MD, Pham DV, Le LP, Gia Le LH, Minh Tran LB, Dang Huynh MD, et al. Recurrent PROC and novel PROS1 mutations in Vietnamese patients diagnosed with idiopathic deep venous thrombosis. Int J Lab Hematol 2021; 43(2): 266–72. [CrossRef]
17. Seo JY, Lee KO, Kim SH, Oh D, Kim DK, Kim HJ. The genomic architecture of the PROS1 gene underlying large tandem duplication mutation that causes thrombophilia from hereditary protein S deficiency. Gene 2014; 547(2): 295–9. [CrossRef]
18. Jang MA, Kim SH, Kim DK, Kim HJ. A novel nonsense mutation Tyr301* of PROS1 causing protein S deficiency. Blood Coagul Fibrinolysis 2015; 26(2): 223–4. [CrossRef]
19. Menezes J, Ventura C, Costa JM, Parreira E, Romão L, Gonçalves J. PROS1 novel splice-site variant decreases protein S expression in patients from two families with thrombotic disease. Clin Case Rep 2017; 5(12): 2062–5. [CrossRef]
20. Xu J, Peng G, Ouyang Y. A novel mutation Gly222Arg in PROS1 causing protein S deficiency in a patient with pulmonary embolism. J Clin Lab Anal 2020; 34(4): e23111. [CrossRef]

21. Wang X, Tang N, Wang X, Lu Y, Yang J. PROS1 IVS10+5G>A mutation causes hereditary protein S deficiency in a Chinese patient with pulmonary embolism and venous thromboembolism. Thromb Res 2019; 174: 1–4. [CrossRef]
22. Ansari N, Najafi S, Shahrabi S, Saki N. PEAR1 polymorphisms as a prognostic factor in hemostasis and cardiovascular diseases. J Thromb Thrombolysis 2021; 51(1): 89–95. [CrossRef]
23. Zhan Q, Ma X, He Z. PEAR1 suppresses the proliferation of pulmonary microvascular endothelial cells via PI3K/AKT pathway in ALI model. Microvasc Res 2020; 128: 103941. [CrossRef]
24. Izzi B, Gianfagna F, Yang WY, Cludts K, De Curtis A, Verhamme P, et al; Moli-family Investigators. Variation of PEAR1 DNA methylation influences platelet and leukocyte function. Clin Epigenetics 2019; 11(1): 151. [CrossRef]
25. Nie XY, Li JL, Qin SB, Fu Y, Liang GK, Shi LW, et al. Genetic mutations in PEAR1 associated with cardiovascular outcomes in Chinese patients with acute coronary syndrome. Thromb Res 2018; 163: 77–82. [CrossRef]
26. Keramati AR, Yanek LR, Iyer K, Taub MA, Ruczinski I, Becker DM, et al. Targeted deep sequencing of the PEAR1 locus for platelet aggregation in European and African American families. Platelets 2019; 30(3): 380–6. [CrossRef]
27. Yabe M, Matsubara Y, Takahashi S, Ishihara H, Shibano T, Miyaki K, et al. Identification of ADRA2A polymorphisms related to shear-mediated platelet function. Biochem Biophys Res Commun 2006; 347(4): 1001–5. [CrossRef]
28. Chen X, Liu L, He W, Lu Y, Ma D, Du T, et al. Association of the ADRA2A polymorphisms with the risk of type 2 diabetes: a meta-analysis. Clin Biochem 2013; 46(9): 722–6. [CrossRef]
29. Totomoch-Serra A, Muñoz ML, Burgueño J, Revilla-Monsalve MC, Perez-Muñoz A, Diaz-Badillo Á. The ADRA2A rs553668 variant is associated with type 2 diabetes and five variants were associated at nominal significance levels in a population-based case-control study from Mexico City. Gene 2018; 669: 28–34. [CrossRef]
30. Akar N. Factor V 1691 G-A mutation distribution in a healthy Turkish population. Turk J Haematol 2009; 26(1): 9–11.