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RESEARCH ARTICLE

Evaluation of Protein C Gene Polymorphism in Patients with Diagnosis of Pulmonary embolism in Turkish Population

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ABSTRACT

Objective: Pulmonary embolism is usually a complication of deep vein thrombosis (DVT) and develops as a result of obstruction of pulmonary artery and/or branches with pieces that ruptured from the DVT of the leg. Pulmonary embolism and DVT is also referred as venous thrombo-embolism (VTE), because two events often remain together. In the studies, it was found that protein C (PROC) deficiency is a risk factor for pulmonary embolism. In this study, we aimed to evaluate the association between pulmonary embolism and PROC gene -1654C>T polymorphism in Turkish population.

Methods: The DNAs of 114 pulmonary embolism cases and 120 healthy controls have been analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) to evaluate the relation between PROC gene -1654C>T polymorphism and pulmonary embolism in our study. Statistical analyses were performed by using chi-square and analysis of variance tests.

Results: The proportion of individuals with CT genotype carrying polymorphic T allele as heterozygous form was 38.7% in the control group and 21.9% in the pulmonary embolism cases (p=0.047). When demographic and clinical characteristics of cases compared with PROC gene -1654C>T polymorphism, it was observed that the changes in chest CT ratios could be associated with -1654C>T polymorphism (p=0.017).

Conclusion: As a result, individuals with CT genotypes carrying the polymorphic T allele as heterozygous form have a lower risk of developing pulmonary embolism.

Key words: PROC; VTE; pulmonary embolism; polymorphism

INTRODUCTION

Venous thromboembolism (VTE) is a common hematologic disease that can occur due to several causes. Although it can be fatal, it is a preventable disease [1, 2]. This disease, formerly seen rather rarely, has become the most common vascular disorder, because humans started to live sedentary life since the first published case of VTE in 13th century. VTE bears significance, because it can involve different locations, can have a course with repeating attacks and can lower the quality of life with late onset complications as well as in terms of its unfavorable impacts on national health costs [2]. VTE presents itself most commonly as deep vein thrombosis (DVT), or as pulmonary embolism [2, 3]. Less often, development of thrombus can be seen in other vascular structures. Thrombi, mostly occurring in deep veins of lower extremities, can cause local complaints related to relevant extremity and sometimes it can shear off and join circulation, reaching pulmonary arteries or their branches. They can then obstruct these vessels, leading to pulmonary embolism, which is a potentially fatal complication. Lower extremity vein thrombosis, that is usually symptom-free, accompanies at least 70% of the cases with pulmonary embolism [4, 5]. It has been suggested that ethnicity along with genetic risk factors could play important role in development of pulmonary embolism. It is acknowledged that Factor V Leiden and coagulation factor II gene mutations are the two major hereditary factors associated with VTE in Europe and the USA [6, 7]. However, these mutations are much less common in Europe compared to Asian societies [8]. It has been shown that molecular mechanism

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of pulmonary embolism varies in different societies [9].

Protein C (PROC) is a vitamin K-dependent serine protease produced and secreted by hepatic cells as a zymogen. Along with cofactor S (PS), activated PROC has a proteolytic effect on factor Va and factor VIIIa in regulation of blood clotting pathway. In 1981, it was first described by Griffin and his colleagues that hereditary PROC deficiency leads to hypercoagulability condition. As an autosomal dominant trait, hereditary PROC deficiency can arise from several distinct mutations in the PROC gene. PROC variants occurring as the result of these genetic changes lead to severe intracellular impairments and ineffective PROC release [10, 11] or non-functional PROC release [12]. Clinical definition of PROC deficiency varies because of the increased risk of VTE complication without any apparent reason in young adults [12]. Prevalence of hereditary PROC deficiency was estimated at 0.2-0.5% [13, 14]. Heterozygous mutation remains asymptomatic for life in many individuals with PROC deficiency [9, 14].

The PROC gene, comprising nine exons and eight introns, is located on chromosome 2q14-21 and spans 11 kb [15]. Precursor PROC protein consists of a preproleader sequence of 42 amino acids, a light chain of 155 amino acids, a connecting dipeptide of Lys-Arg linkage and a heavy chain of 262 amino acids [16]. PROC gene neighboring sequence contains several transcriptional regulatory regions. Distinct polymorphic loci were identified on promoter region of the human PROC gene [17]. It was shown that polymorphic regions of the PROC gene (-1654C>T, -1641A>G and -1476A>T) were associated with DVT incidence in some countries [18, 19]. In a study conducted on Chinese population, three SNPs were investigated in the PROC gene in pulmonary embolism. Genotype frequencies of these three SNP regions (-1654C>T, -1641A>G and -1476A>T) along with allele frequencies of 1654C>T polymorphism showed significant difference between embolism group and control group. These findings suggested that TT phenotype of -1654C>T polymorphism could be associated with tendency to develop pulmonary embolism in Chinese population (OR 2.245, %95 CI, 1.252-4.027). It is found that T allele is a risk factor for pulmonary embolism and C allele acts as a protective factor [9].

We aimed to investigate the relation of the PROC gene -1654C>T polymorphism with pulmonary embolism in Turkish population.

METHODS

Study Group

One hundred and fourteen cases aged between 29 and 81 who were admitted to Gaziosmanpasa University Emergency Medicine Clinic with pulmonary embolism and 120 healthy controls without any personal and family history of pulmonary embolism who were in similar age group were included in the study. The confirmation of pulmonary embolism was made with thorax computed tomography pulmonary angiography (CTPA) by emergency physicians and radiologists. The diagnostic criteria for pulmonary embolism include the following: 1. Arterial occlusion with failure to enhance the entire lumen due to a large filling defect; the artery may be enlarged compared with adjacent patent vessels. 2. A partial filling defect surrounded by contrast material, producing the “polo mint” sign on images acquired perpendicular to the long axis of a vessel and the “railway track” sign on longitudinal images of the vessel. 3. A peripheral intraluminal filling defect that forms acute angles with the arterial wall. The cases who were highly suspected of pulmonary embolism were taken into the study according to CTPA results. The cases who confirmed pulmonary embolism with Ventilation/Perfusion Scintigraphy were out of the study. Control group was free from VTE, coronary artery disease, malignancy, pregnancy, previous surgery and stroke. Controls with family history of any evidence for thrombosis and women controls with prior history of abortions or other obstetric complications were excluded from the study. All participants, cases and healthy controls, were of Turkish origin, from the inner Central Black Sea region of Turkey. Written informed consent was obtained from all cases and controls regarding the inclusion to the study. Approval from Scientific Research Ethical Committee of Gaziosmanpasa University Medical Faculty was obtained.

Genotype Analysis

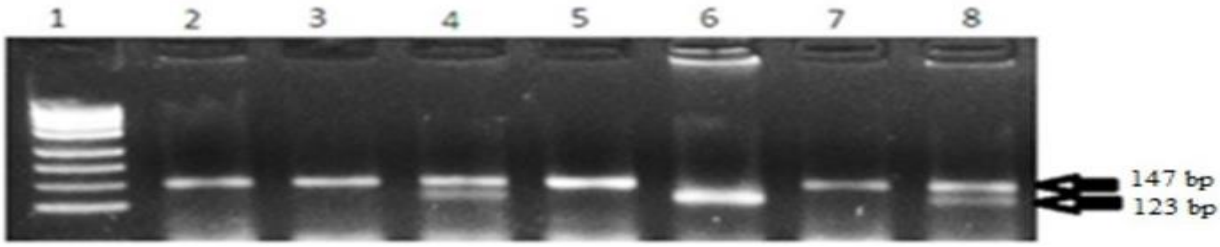
Blood samples were taken from study cases into standard 5 mL EDTA tube. Study was entirely conducted in Gaziosmanpasa University Medical

Biology and Genetics Laboratory. Isolation of DNA from blood was performed with commercially available DNA isolation kit. DNA samples were stored at -20°C. The PROC gene -1654C>T (C2405T, rs1799808) polymorphism analysis was done with polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). PCR was performed using F: 5'GGG CAA AAA TGT CCC CAT CTG AA3' and R: 5'AGC CCA CCT CTG CCC ACC AAG AAT G3' primers. PCR program was adjusted so that first denaturation occurred at 94°C 4 min, denaturation at 94°C 45 sec, binding at 65°C 40 sec, elongation at 72°C 30 sec, final elongation at 72°C 5 min and number of cycle was 35. PCR product of 147 bp was obtained after multiplication. In total, 15µl restriction mixture, containing Fast Digest Buffer (10×) 1.5 µl, BseGI (BtsCI) enzyme (10u/µl) 0.5 µl, dH2O 2.85 µl, BSA 0.15 µl, and PCR product 10 µl, was prepared for cutting the PCR products with restriction enzyme. After leaving PCR products with restriction enzyme BseGI (BtsCI) at 55°C for 12 hours, -1654C>T polymorphism was evaluated. C allele remained uncut with restriction enzyme, while T allele was broken into two fragments of 123 bp and 24 bp (Figure 1).

Statistical Analyses

Statistics Package Program for Social Sciences (SPSS) version 20.0 and Openepi 3.01 (www.openepi.com) software programs were used in order to determine statistical analysis. Results were given as mean plus (±) standard deviation. Relation between the -1654C>T polymorphism and clinical and demographic characteristics was analyzed using chi-square (χ^2), Fisher exact or variant analysis (ANOVA tests). Odds Ratio (OR) and 95% Confidence Interval (CI) were used for detecting the risk factors. All p values were two-tailed and p values less than 0.05 were regarded as statistically significant. For the detected genotype frequencies, occurrence of deviation from Hardy-Weinberg equilibrium and presence of consistency in controls were identified with chi-square test.

Figure 1. RFLP results of PROC gene -1654C>T polymorphism determined by BseGI (BtsCI) restriction endonuclease. See Examples 2, 3, 5, 7 homozygous dominant (CC); 6 homozygous recessive (TT); 4, 8 heterozygous (CT), 1 pUC19 DNA/MspI (HpaII) marker



RESULTS

In this study, in order to determine the association between the PROC gene -1654C>T polymorphism and pulmonary embolism, 114 cases with average age of 58.27± 10.629 (minimum: 29, maximum: 81) who had pulmonary embolism and 120 healthy control subjects with average age of 56.02±7.920 (minimum: 43, maximum: 77) were studied. Both familial and non-familial group of pulmonary embolism cases, who were admitted from Tokat province and its vicinity to Gaziosmanpasa University Medical Faculty, Department of Emergency Medicine were included into the study. Age and gender of the case and control groups were consistent (Table 1).

Frequencies of genotype and allele in cases and controls are shown in Table 2. When C and T allele frequencies of the cases (82.1% and 17.9% respectively) and controls (76.7% and 23.3% respectively) were compared to each other, there was no statistically significant difference (p=0.094), while frequencies of CC, CT and TT genotypes showed statistically significant differences between the cases and the controls (p=0.047). The proportion of individuals with CT genotype carrying polymorphic T allele as heterozygous form was 38.7% in the control

group and 21.9% in the pulmonary embolism cases. A more significant relation was seen when the case and the control genotypes were compared CC vs TT+CT (p=0.029) (Table 2). Frequency of CT+TT genotype was higher in the controls compared to the cases (41.7% vs 28.1%), therefore this finding suggested that these genotypes could be protective against the disease.

Assessment of demographic and clinical characteristics of the pulmonary embolism cases according to the PROC gene -1654C>T polymorphism is presented in Table 3. χ^2 and variance analysis revealed that there was no statistically significant relationship between age, gender, coexisting disease (surgical, coronary artery disease, diabetes, hypertension, cerebrovascular disease, malignancy), ECG abnormalities (RBBB, sinus tachycardia, S1Q3T3, AF, ischemia), smoking and the PROC gene -1654C>T polymorphism. However, a significant relationship was found between thorax CT (right main bronchus, right distal, left main bronchus, left distal, both main bronchi and both distal) data and the PROC gene –1654C>T polymorphism (p=0.017) (Table 3). All cases with embolism in left main bronchus had CC genotype, while all of those with embolism in left distal had CT genotype.

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Table 1. Statistical evidence of gender and mean age of individuals in the pulmonary embolism cases and control groups included in the study

	Pulmonary Embolism Case Group (N=114)	Control Group (N=120)	P Value
Gender, N (%)			0.312
Female	55 (48.2%)	50 (41.7%)	
Male	59 (51.8%)	70 (58.3%)	
Mean Age, Years	58.27±10.629	56.02±7.920	0.066

Data were analyzed by analysis of variance or χ2 test. Mean plus standard deviation values are presented for age.

Table 2. Genotype and allele distribution of *PROC* gene 1654C>T polymorphism for pulmonary embolism case group and the control group

	Case Group N=114 (%)	Control Group N=120 (%)	X^2	P Value	Or(%95cl)
GENOTYPE					
CC	82 (71.9)	70 (58.3)			
CT	25 (21.9)	44 (36.7)	**6.10**	**0.047**	
TT	7 (6.1)	6 (5.0)			
CC: CT+TT	82 (71.9) : 32 (28.1)	70 (58.3) : 50 (41.7)	**4.75**	**0.029**	**0.55 (0.31-0.94)**
CC+CT: TT	107 (93.9) : 7(6.1)	114 (95.0) : 6 (5.0)	0.14	0.703	1.24 (0.39-4.05)
ALLELE					
C	189(82.9)	184 (76.7)			
T	39 (17.1)	56 (23.3)	2.80	0.094	0.68 (0.43-1.07)

Data were analyzed by χ2 test. *PROC*: Protein C. The results that are statistically significant are typed in bold.

DISCUSSION

Pulmonary embolism, substantially originated from DVT in lower limbs, is a disease with a spectrum ranging from asymptomatic and incidentally detected embolism to severe embolism which causes death. Acute pulmonary embolism may occur in a rapid and unexpected manner, and its diagnosis can be challenging. Treatment can lower the risk of death and proper primary prophylaxis is usually effective [20].

PROC is a vitamin K-dependent plasma glycoprotein. Mild and severe genetic disorders of this protein were associated with venous thrombosis and neonatal purpura fulminans, respectively. Recent studies showed that APC inactivates factors Va and VIII to prevent thrombin formation [21]. Individuals with PROC deficiency carry high risk for DVT and pulmonary embolism.

In this study, we investigated the relation between the PROC gene -1654C>T polymorphism and pulmonary embolism. A statistically significant

relation was found between the PROC gene -1654C>T polymorphism and pulmonary embolism (p=0.047). The frequency of CC genotype in case group was higher than the frequency of CT and TT genotypes in our study, and this finding suggests that those who carry the polymorphic CT + TT genotypes have a lower risk of developing pulmonary embolism than those who carry the wild CC genotype. In the PROC gene, 987 SNP were detected (http://www.ncbi.nlm.nih.gov/clinvar, Accessed on September 14, 2015). One of these included -1654C>T polymorphism, which we studied. This polymorphism was first detected in a case with pulmonary embolism by Hoshi et al. and was first defined as -154C>T [22]. Five cis elements that regulate PROC expression were identified [23]. Of these, sequences between -88 and +45 is the main positive promoter, between -419 and -298 is weak positive promoter and between -144 and 88 is weak negative promoters. The exact importance of the C>T change in -154th nucleotide is not clear yet. However, possible regulatory impact of promoter polymorphism on PROC gene expression can affect the penetrance of individuals with heterozygote deficiency and cause Type II deficiency [22].

Table 3. Demographic and clinical characteristics of pulmonary embolism cases included in the study evaluated according to *PROC* gene -1654C> T polymorphism.

		PROC –1654 C>T			
Characteristics	**Total** n=114	**CC** n=82	**CT** n=25	**TT** n=7	**P value**
Gender, Male/Female, N (%)	59/55 (51.8/48.2)	42/40 (51.2/48.8)	15/10 (60.0/40.0)	2/7 (28.6/71.4)	0.333
Age, Years	58.27±10.629	59.38±10.452	54.92±10.004	57.29±13.708	0.180
Wbc, Per Mm^3	9947.9±2836.5	10290.5±2861.6	8861.8±2326.6	9952.8±3581.5	0.098
Rdw, %	16.34±2.511	16.19±2.446	16.63±2.280	17.10±3.923	0.541
Htc, %	38.54±4.131	38.92±4.346	37.70±3.573	37.28±3.153	0.320
Surgery, N (%)	3 (2.6)	3 (3.7)	0	0	0.548
Smoking, N (%)	64 (56.1)	44 (53.7)	17 (68.0)	3 (42.9)	0.344
Comorbidity, N (%)					
Coronary Artery Disease	40 (35.1)	30 (36.6)	7 (28.0)	3 (42.9)	0.664
Diabetes Mellitus	41 (36.0)	27 (32.9)	10 (40.0)	4 (57.1)	0.393
Hypertension	61 (53.5)	42 (51.2)	14 (56.0)	5 (71.4)	0.566
Chronic Renal Failure	15 (13.2)	12 (14.6)	2 (8.0)	1 (14.3)	0.689
Cerebrovascular Disease	8 (7.0)	6 (7.3)	2 (8.0)	0	0.750
Malignancy	10 (8.8)	7 (8.5)	3 (12.0)	0	0.605
ECG Abnormalities, N (%)					
Rbbb	19 (16.7)	16 (19.5)	3 (12.0)	0	0.321
Sinus Tachycardia	39 (34.2)	27 (32.9)	8 (32.0)	4 (57.1)	0.417
S1q3t3	31 (27.2)	21 (25.6)	7 (28.0)	3 (42.9)	0.613
Af	29 (25.4)	20 (24.4)	7 (28.0)	2 (28.6)	0.918
Ischemia	14 (22.3)	11 (13.4)	2 (8.0)	1 (14.3)	0.760
Thorax Ct					**0.017**
Right Main Bronchus	20 (18.5)	14 (17.9)	5 (21.7)	1 (14.3)	
Right Distal	29 (26.9)	20 (25.6)	7 (30.4)	2 (28.6)	
Left Main Bronchus	6 (5.6)	6 (7.7)	0	0	
Left Distal	4 (3.7)	0	4 (17.4)	0	
Bilateral Main Bronchus	45 (41.7)	36 (46.2)	6 (26.1)	3 (42.9)	
Bilateral Distal	4 (3.7)	2 (2.6)	1 (4.3)	1 (14.3)	

Data were analyzed by analysis of variance or χ2 test. Mean plus standard deviation values are presented for age, WBC, RDW, HTC. AF: Atrial fibrillation, CT: Computed tomography, ECG: Electrocardiography, HTC: Haematocrit, PROC: Protein C, RBBB: Right bundle-branch block, RDW: Red blood cell distribution width, WBC: White blood cell. The results that are statistically significant are typed in bold.

There are two studies in the literature that examined the relation between PROC gene -1654C>T polymorphism and pulmonary embolism. Both of these studies were conducted in China and they revealed quite the opposite of our results. First study was conducted by Zhang et al. on 63 cases with pulmonary thromboembolism and 86 control cases; and they found that -1654C>T polymorphism T allele (2405T) increases the risk for pulmonary tromboembolism, whereas C allele is a potential protective factor [24]. Second study was conducted by Zhu et al. on 110 cases with pulmonary embolism and 190 control cases [9]. This study investigated three SNPs in the PROC gene. Genotype frequencies of these three SNP regions (-1654C>T, -1641A>G and -1476A>T) along with C and T allele frequencies of 1654C>T polymorphism showed significant difference between embolism group and control group. Contrary to our results, these findings showed that TT genotype of -1654C>T polymorphism constitutes a risk factor for pulmonary embolism (OR 2.245, 95%CI, 1.252-4.027) [10].

Relation between the PROC gene -1654C>T polymorphism and VTE has been studied by many researchers. Spek et al. showed that for the PROC gene C(–1654)T, A(–1641)G, and A(–1476)T polymorphic regions, CC/GG/TT haplotype is related to decreased PROC concentration and hence venous thrombosis [18]. Aiach et al. investigated the relation between the PROC gene 1654C>T along with –1641A>G polymorphic regions and venous thrombosis in 242 VTE cases and 349 control cases in a French population [19]. They reported that risk of thrombosis is high in young carriers with CG haplotype and therefore CG haplotype is related to low PROC levels. Finally, Pomp et al. (2009), investigated the effect of two polymorphisms (2405C>T and 2418A>G) in the PROC gene promoter region on venous thrombosis and plasma levels of PROC, and found that CC/GG genotype is associated with lower PROC levels and it increases the risk of thrombosis 1.3 fold (95% CI 1.09-1.48) [25]. The results of these studies, conducted on European population, comply with our results.

Ethnical origin can play crucial role on genetic risk factors for several diseases, including pulmonary embolism. Polymorphisms in Factor V Leiden and coagulation factor II genes are the two major hereditary factors associated with venous thrombosis in the USA and Europe. However, these changes are very uncommon in Asian population [9]. It is seen that molecular mechanism of pulmonary embolism can vary in different populations.

Our study is the first research that is conducted on Turkish population in regard to investigate the relation between the PROC gene -1654C>T polymorphism and pulmonary embolism. According to the findings we obtained in this study, the proportion of individuals with CT genotype carrying polymorphic T allele as heterozygous form was 38.7% in the control group and 21.9% in the pulmonary embolism cases. Our findings show that further genetic tests are needed in cases with pulmonary embolism. For this purpose, larger studies should be conducted.

In conclusion, a statistically significant relation was found between the PROC gene -1654C>T polymorphism and pulmonary embolism. The limitation of the present study is the absence of PROC levels of cases and controls. It would be better to measure PROC levels of all subjects in order to see the effect of -1654C>T polymorphism on PROC levels in our study group. More detailed establishment of the relation between the PROC gene -1654C>T polymorphism and pulmonary embolism can enable prediction of the risk factors for pulmonary embolism and prevention of the disease.

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