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ORIGINAL ARTICLE

Investigation of the Frequency of Thrombophilic Gene Mutations in Patients with Venous Thromboembolism in Eastern Türkiye

Türkiye'nin Doğu Anadolu Bölgesi'nde Venöz Tromboembolizm olan Hastalarda Trombofilik Gen Mutasyonlarının Sıklığının Araştırılması

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ABSTRACT

Objectives: Polymorphisms in the thrombophilia genes such as Factor V Leiden (FVL), Prothrombin G20210A and methylenetetrahydrofolate reductase (MTHFR) cause genetic predisposition to thrombophilia. The regional incidence of these polymorphisms varies. The aim of our study is to evaluate the regional frequency of the most common single nucleotide polymorphisms of these thrombophilia genes.
Methods: In this retrospective study, patients diagnosed with VTE in our center were included in the study. The presence of FVL, Prothrombin G20210A, MTHFR C677T, MTHFR A1298C, plasminogen activator inhibitor (PAI)-1, β-Fibrinogen, Factor XIIIA (V34L) and Glycoprotein IIIA (L33P) were investigated in blood samples obtained from the patients via pyrosequencing technique, and the association of genotype disorders was also evaluated.
Results: Eight genotypes were analyzed in 2000 patients whose thrombophilia panel was studied in our clinic. The frequency of heterozygosity was 4.6% for the Factor II G20210A polymorphism, the homozygosity was 0.4%for the Factor V Leiden polymorphism, 7.6% for MTHFR C677T, 48.1% and 15.7for MTHFR A1298C, 38.8% and 13%for PAI-1, 30.3% and 4%for β-Fibrinogen, 23.3% and 2.4% for Factor XIIIA (V34L) and 17.7% and 1.5% for Glycoprotein IIIA (L33P) respectively.
Conclusions: Factor V Leiden and prothrombin G20210A mutations were found at a higher rate in our region compared to other regions in the west.

Key words: Thrombophilia, Venous Thromboembolism, Gene Mutation

ÖZ

Amaç: Faktör V Leiden (FVL), protrombin G20210A ve metilentetrahidrofolat redüktaz (MTHFR) gibi trombofili genlerindeki polimorfizmler, trombofili için genetik yatkınlığa neden olur. Bu polimorfizmlerin bölgesel insidansı değişiklik gösterebilmektedir. Çalışmamızın amacı, bu trombofili genlerinin en sık görülen tek nükleotid polimorfizmlerinin bölgesel sıklığını değerlendirmektir.
Yöntemler: Retrospektif olarak dizayn edilen bu çalışmada merkezimizde VTE tanısı alan hastalar araştırmaya dahil edildi. Hastalardan elde edilen kan örnekleri ile FVL, protrombin G20210A, MTHFR C677T, MTHFR A1298C, plazminojen aktivatör inhibitörü (PAI)-1, β-Fibrinojen, Faktör XIIIA (V34L) ve Glikoprotein IIIA (L33P) varlığı pirosekanslama yöntemi ile araştırıldı ve genotip bozukluklarının birlikteliği de ayrıca değerlendirildi.
Bulgular: Kliniğimizde trombofili paneli çalışılan 2000 hastada sekiz genotip analiz edildi. Faktör II G20210A polimorfizmi için heterozigotluk sıklığı %4.6, Faktör V Leiden polimorfizmi için homozigotluk %0.4, MTHFR C677T için %7.6, MTHFR A1298C için %48.1 ve %15.7, PAI-1 için %38.8 ve %13 , β-Fibrinojen için %30.3 ve %4, Faktör XIIIA (V34L) için %23.3 ve %2.4 ve Glikoprotein IIIA (L33P) için % 17.7 ve %1.5 bulunmuştur.
Sonuç: Bölgemizde Faktör V Leiden ve protrombin G20210A mutasyonları batıdaki diğer bölgelere göre daha yüksek oranda bulundu.

Anahtar kelimeler: trombofili, venöz tromboembolizm, gen mutasyonu

Introduction

Venous thromboembolism (VTE) is an important cause of morbidity and mortality today. Thrombophilia, one of the causes of VTE, is used to define hemostasis diseases that cause thromboembolism. Hereditary and acquired causes play a role in the etiology of thrombophilia(1). Factor V Leiden (FVL) and prothrombin G20210A gene mutation, which are the most common hereditary risk factors for thrombosis, carry a high risk for VTE(2).

The frequency of mutations in thrombophilia genes varies according to populations and ethnicities. There are different studies on thrombophilia from Turkiye and different data have been published(3, 4). However, Erzurum and its close region are different from other regions of Turkiye, where consanguineous marriages are more frequent and consist of different ethnic communities(5). Erzurum is the region where Caucasian Communities live and communicate most frequently in our country. In addition, this region is the intersection point of the Black Sea, South, East and Central Anatolia Regions in our country. It has hosted many ethnic origins throughout history. The aim of this study is to examine whether the prevalence and diversity of thrombophilia genes in societies in the Eastern Anatolia Region differ from other regions of our country and the world.

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Materials and Methods

Study Design and Population

Patients were included in the study if they had one or more of the following criteria according to the National Hematology Hereditary Thrombophilia Diagnosis and Treatment Guidelines, patients were screened retrospectively between March 2014 and June 2015;

- who had the first idiopathic thrombosis attack at the age of 40 and before,
- with a family history of thrombosis,
- children presenting with purpura fulminans,
- pregnant women at risk of venous thrombosis,
- those with a history of recurrent idiopathic/minor triggering VTE.

The presence of FVL, prothrombin G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T, MTHFR A1298C, plasminogen activator inhibitor (PAI)-1, β-Fibrinogen, Factor XIIIA (V34L) and Glycoprotein IIIA (L33P) were investigated in the blood taken from the patients in the genetics laboratory; they were classified according to age, gender, clinics, and the association of genotype disorders was evaluated. The patients were divided into five groups according to their age (group1: below 17, group2: 18-34, group3: 35-49, group4: 50-64, group5: over 64). The study was approved by Atatürk University Clinical Research Ethics Committee (Decision No: 8/14). All patients participating in the study were informed about the study and informed consent forms were signed and their consents were obtained.

Extraction of Genomic DNA

In order to determine the relevant polymorphisms, 2 ml peripheral venous blood samples were taken from the patients in EDTA tubes. Genomic DNAs were obtained from blood samples using the QIAGEN® DNA isolation kit according to the kit instructions. DNA concentrations were measured using the Nanodrop System (Thermo Fisher Scientific, MA, USA) and Qubit dsDNA HS kits (Life Technologies, Gaithersburg, USA).

DNA Amplification

Genomic DNA was amplified using Suspected partial filling defect in the left transverse sinus Polymerase Chain Reaction (PCR) method and specific primers. The PCR mixture given in Table 1 was used for the PCR method. The amplification process was completed for use in pyrosequencing using the prepared PCR mixture and the PCR procedure given in Table 2 in a thermal cycler (SensoQuest Lab-cycler, GmbH, Hilden, Germany).

Detection of Mutations by Pyrosequencing

Sequence analysis with the QIAGEN® Pyromark Q24 system with specific primers for Factor II g.20210G>A, Factor V Leiden, MTHFR C677T, MTHFR A1298C, PAI-1, B-Fibrinogen, Factor XIIIA (V34L) and Glycoprotein IIIA (L33P) polymorphisms was performed. The pyrosequencing results were analyzed and evaluated with Pyromark Q24 Advanced Software.

Statistical Analysis

IBM (SPSS) Statistics v.12.0 (SPSS Inc., Chicago, IL) package program was used in the analysis of the data. Continuous variables in the study are presented as mean ± standard deviation, median (1st quartile-3rd quartile), minimum and maximum. Categorical variables were expressed as numbers and percentages. The Shapiro-Wilk test was used to assess whether the distribution of numerical variables conformed to the normal distribution. Comparisons between groups were evaluated using the chi-square test for categorical variables, independent sample Student's t-tests for normally distributed continuous variables, and the Mann-Whitney U test for two independent group comparisons for variables when the assumption of normality was not met. Correlations were evaluated using Pearson's or Spearman's correlation tests. Kruskal-Wallis test was performed to determine whether there were differences in genetic parameters between departments. The statistical significance level was taken as $p < 0.05$.

Results

Factor V Leiden, prothrombin G20210A, MTHFR C677T, MTHFR A1298C, PAI-1, β-Fibrinogen, Factor XIIIA (V34L) and Glycoprotein IIIA (L33P) genotypes were analyzed in 2000 patients whose thrombophilia panel was studied in our clinic. In this study, blood results from 20 different clinics were analyzed. The mean age of the patients was 34.7±10.8 (2-87) years. 50% of the patients were between the ages of 18-34 and 78.5% (1570) were women. 48.5% of the blood samples were sent from neurology clinics and 29% from gynecology and infertility clinics (Table 3).

When the distribution of the frequency of genotypes determined by the thrombophilia panel is examined; it was determined that 4.6% for Factor II G20210A polymorphism and 8.8% for Factor V Leiden polymorphism carried heterozygous mutant genotypes (Table 4). 16.5% of people whose genetic thrombophilia panel was examined had only one genotype disorder. When the coexistence of two genotype disorders was examined, MTHFR A1298C and PAI-1 were found in 33.2%. The association of Factor V Leiden and Factor II was 0.8%.

When genotype disorders were examined according to clinics, there was a disorder in all 8 genotypes examined in blood samples from internal medicine, infertility, gynecology, cardiovascular surgery, neurology, organ transplantation and medical genetics clinics (Table 5).

When genotype disorder was evaluated according to gender, no difference was found between genders. When genotype disorder was evaluated according to age groups, no difference was found between age groups ($p>0.05$).

Table 1. PCR mixture

Reaction mixture	Amount
PCR Master Mix (Qiagen GmbH, Hilden, Germany)	18.5 µl
Forward primer (F)	2 µl
Reverse primer (R)	2 µl
Taq DNA Polymerase (5U/µl)	0.5 µl
Genomic DNA	10 µl
H_2O	17 µl
Total volume	50 µl

Table 2. PCR procedure for thermal cycler

Reaction step	Temperature °C	Time	Cycle
Initial denaturation	95	15 min	1
Denaturation	94	1 min	32
Primer annealing	56	1 min	
Primer extension	72	80 min	
Last extension	72	15 min	1

Table 3. Distribution of the blood samples examined according to the clinics

	Clinics	Number of patients (n)	%
1	Neurosurgery	1	0.1
2	Dermatology	4	0.2
3	Internal medicine	132	6.6
4	Physical therapy and rehabilitation	2	0.1
5	Infertility	235	11.8
6	Cardiology	5	0.3
7	Ear nose throat diseases	2	0.1
8	Neurology	969	48.5
9	Organ transplantation	77	3.9
10	Psychiatry	2	0.1
11	Obstetrics and gynecology	343	17.2
12	Cardiovascular surgery	43	2.2
13	Pediatrics	43	2.2
14	Chest diseases	11	0.6
15	Maternal medicine	19	1.0
16	General surgery	5	0.3
17	Ophtalmology	2	0.1
18	Medical oncology	1	0.1
19	Medical genetic	100	5.0
20	Infectious diseases	4	0.2
	Total	2000	100

Table 4. Distribution of the Frequency of Genotypes Determined by the Thrombophilia Panel

	Normal		Positive		Homozygote		Heterozygote		Total
	n	%	n	%	n	%	n	%	(n)
Proth-tombin G20210A	1908	95.4	92	4.6	-		92	4.6	2000
Factor V Leiden	1816	90.8	184	9.2	7	0.4	177	8.8	2000
MTHFR C677T	1103	55.2	898	44.8	151	7.6	747	37.2	2000
MTHFR A1298C	719	36.2	1270	63.8	313	15.7	957	48.1	1989
PAI-1	962	48.2	1036	51.8	261	13	775	38.8	1998
β -Fibri-nogen	1313	65.7	687	34.3	80	4	607	30.3	2000
Factor XIIIA (V34L)	1483	74.3	513	25.7	47	2.4	466	23.3	1996
Glycopro-tein IIIA (L33P)	1616	80.8	383	19.2	30	1.5	353	17.7	1999

Table 5. Genotypes according to clinics

Clinics	PROTHROMBIN G20210A	FACTOR V G1691A (Leiden)	MTHFR C677T	MTHFR A1298C	PAI-1 (5G/4G)	B-FIBRINOGEN (455G>A)	F XIIIA (V34L)	GPIIIA (L33P)
Internal medicine	12	18	55	56	66	45	32	24
Infectious dise-ases	1		4	1	3	2	2	1
Infertility	7	20	99	114	150	86	65	45
Obstetrics and gynecology	13	29	161	161	167	125	90	75
Cardiovascular surgery	9	15	16	15	29	20	8	12
Neurology	40	78	443	485	481	326	247	181
Organ transplan-tation	3	4	42	38	38	16	19	11
Psychiatry	1	1		2	1	1	1	
Medical genetic	7	11	40	52	53	34	26	20
Pediatrics		6	16	15	22	15	11	6
Chest diseases		1	3	3	4	2	1	1
Maternal me-dicine		3	7	6	12	7	6	3
Dermatology			3	3	3	2	1	1
Physical therapy and rehabilitation			1	1	2		1	
General surgery			3	3	4	3	1	
Ophtalmology			1	1	2	1		
Cardiology			3	2	2	1	1	2
Ear nose throat diseases				1				1
Neurosurgery					1	1	1	1

Discussion

Thrombophilia is a term used to describe the conditions in which the tendency to clot in the blood increases. Thrombosis development is multifactorial, and it is known that many acquired (secondary) and hereditary (primary) factors cause thrombosis by different mechanisms(6).

The frequency of mutations in thrombophilic genes varies according to populations and ethnicities. In the haplotype analysis of the Factor V gene, it is suggested that the FVL mutation originated in the Caucasus about 30.000 years ago. It is estimated that this mutation later spread to Europe and India 10.000 years ago(7). While the FVL mutation is seen around 15% in Caucasian populations, the other highest prevalence rates are in Mediterranean countries, with 12.3% in Jordan, 14.4% in Lebanon, 13.3% in Greece and 12.1% in Cyprus(8). It has been reported to be between 3-8% in European societies(9). While there is no mutation in some populations such as Japanese, Chinese, African and Native American populations(10), it has been reported in around 1.3% of populations in the Indian Panjabi Region(11). These distributions are important for our country, which is at the crossroads of population migration, and especially for the Erzurum Region where the study was conducted. For this reason, it will contribute to the formation of diagnosis and treatment protocols according to regions by investigating the variation and frequency of this mutation, which has serious consequences, between regions. In some studies conducted regionally in our country, the rate of FVL mutation was found as 7.1% in Ankara(12, 13), 4.2% in Edirne(14), and 4.9% in Sivas(15). In our country, the region where Caucasian Communities live most frequently and are in constant communication is Erzurum province and its surroundings, where the study was conducted. In addition, Erzurum Region is the intersection point of the Black Sea, Southern, Eastern and Central Anatolian Regions in our country and is a region that has hosted many different ethnic origins throughout history. Therefore, this study differs from studies conducted in other regions of our country. The frequency of FVL in the blood analyzed in our study was 9.2%. This rate is higher than the western regions in our country.

Prothrombin G20210A mutation is the second most common cause of prothrombotic polymorphism after FVL disease. Although the prothrombin G20210A polymorphism has different prevalence in the world, it is seen with a frequency up to 3% (16, 17). Prothrombin gene polymorphism is very rare in Africans and Asians(6). The prevalence of prothrombin G20210A polymorphism in Turkiye has been reported as 2.6%(18). In the study conducted by Ayyıldız et al. in Diyarbakir, the frequency of prothrombin G20210A mutation was 1.2% in healthy individuals, while it was reported as 6.5% in VTE patients(19). Oztuzcu et al. reported the prevalence of G20210A polymorphism as 4.6% (3.4% heterozygous, 0.2% homozygous) in the study they conducted in Gaziantep Region(20). In our study, it was determined as 4.6% (heterozygous).

The co-existence of prothrombin G20210A and FVL polymorphisms increases the risk of VTE up to 6-10 times(18). Dolek et al. reported the association of these two polymorphisms at a rate of 1.5% in their study conducted in our country(21). In our study, the association of Factor V Leiden and Factor II was 0.8%.

In MTHFR deficiency, homocysteine cannot be converted to methionine and its level in the blood increases. Several polymorphisms have been identified in MTHFR, but only C677T and A1298C polymorphisms have been confirmed to affect enzyme activity(22, 23). The incidence of MTHFR C677T polymorphism in the community has been reported as 12%(24). In studies conducted in Turkiye, the rate of homozygous mutant genotype was 5% and the rate of heterozygous mutant genotype was 35% in healthy individuals(25). It is seen with a frequency of 24-64% in Europe, 6-64% in North America, and 8-31% in Siberia(26). In our study, the MTHFR C677T mutation was 44.8% (37.2% heterozygous, 7.6% homozygous).

Pregnant, heterozygous carriers or homozygous mutants for MTHFR C677T are at risk for neural tube defects due to hyperhomocysteinemia. In our study, MTHFR C677T polymorphism was one of the two genes with the most genetic disorders in blood from the obstetrics clinic, with a frequency of 19%.

Globally, the MTHFR A1298C allele frequency ranges between 10-70% in Asia, 24-46% in Europe, 13-32% in Africa, and 0-15% in the Americas(26). In studies conducted in our country, this rate has been reported between 50-65%(21). In our study, the MTHFR A1298C mutation was 63.8% (48.1% heterozygous, 15.7% homozygous). The gene polymorphism with the highest rate in our study was the MTHFR A1298C mutation. It has been reported that MTHFR C677T and A1298C mutations increase the risk of venous thrombosis considerably if there are other genetic risk factors, especially factor V Leiden mutation(23, 26). In our study, MTHFR C677T + Factor V Leiden association was 4.6%, and MTHFR A1298C + Factor V Leiden association was 5.25%. In the study of Dölek et al., MTHFR C677T + Factor V Leiden association was reported as 13%, and MTHFR A1298C + Factor V Leiden association as 17%(21). In our region, the association of these two genes with Factor V Leiden was found lower than in other studies. The association of MTHFR C677T + Factor V Leiden + prothrombin G20210A was 0.25%. Kabukçu et al. reported this association as 0.3%, similar to our study(8).

The most common insertion/deletion polymorphism in the PAI-1 gene is a four or five guanine basic (4G/5G) change located 650 bases upstream of the transcription start point within the PAI-1 gene promoter region. This genotype has been shown to be one of the major risk factors for PIHs in pregnant women(27). In studies conducted in our country, the frequency of PAI-1 4G/5G mutation was reported as 45%(20). In our study, the PAI-1 polymorphism was found as 51.8% (38.8% heterozygous, 13% homozygous). PAI-1 (4G/5G) polymorphism was present in 48% of the

blood obtained from obstetrics and gynecology clinics. In addition, in our study, the highest two gene associations were MTHFR A1298C + PAI-1 (32.5%) and MTHFR C677T + PAI-1 (24.4%) polymorphisms.

FXIII deficiency is a rare disease that causes severe bleeding, recurrent miscarriages, and poor wound healing. According to a study conducted on the Lebanese population, the prevalence of heterozygous and homozygous genotypes for Factor XIII V34L mutation was 22.4% and 3.4%, respectively(28). In the study performed by Öztuzcu et al. in our country, the percentage of heterozygous and homozygous Factor XIII V34L mutations was 24.9% and 2.6%, respectively(20). In the authors' study, they noted that the prevalence of V34L carriers (27.5%) in the population analyzed in the Southeastern Region of Turkiye was generally lower than in Caucasians (44.3%) and interestingly similar to that in Blacks and South Asians. In our study, the prevalence of Factor XIIIA V34L polymorphism was 23.3% heterozygous and 2.4% homozygous, similar to the study of Öztuzcu et al.

The GPIIb/GPIIIa complex mediates platelet aggregation by acting as a receptor for fibrinogen. This complex also acts as a receptor in von Willebrand factor and fibronectin(29). The presence of this variant has been associated with the risk of premature acute coronary syndrome and stroke in young Caucasian women(30). In our study, the prevalence of Glycoprotein IIIa (L33P) polymorphism was 19.2% (17.7% heterozygous, 1.5% homozygous) similar to the Caucasian population.

In this study, the frequency of FVL, prothrombin G20210A, MTHFR C677T, MTHFR A1298C, PAI-1, β-Fibrinogen, Factor XIIIA (V34L) and Glycoprotein IIIA (L33P) polymorphisms, which are the causes of genetic mutations that cause thrombophilia, were investigated in the Eastern Anatolia Region of our country and compared with other communities in the world. In addition, this study is the most comprehensive study in the literature according to the distribution of these eight genes together according to the clinics and whether they are together or not. As a result of our study, Factor V Leiden and Factor II were found more common in the Erzurum region compared to the western regions, while the incidence of other related thrombophilia allele mutations was similar to the values reported in the literature.

Heterozygous mutations in the factor V Leiden gene increase the relative risk of VTE 3-8 times while homozygous mutations increase it 80 times (8). Whereas prothrombin G20210A mutation increases the risk of VTE 1-5 times, the co-existence of prothrombin G20210A mutation and Factor V Leiden mutation increases the risk of VTE up to 6-10 times(18). For these reasons, it is important to examine Factor V Leiden and prothrombin G20210A mutations in patients presenting with VTE in Erzurum Region compared to other regions.

Conclusion

There are similarities and differences in the polymorphisms of these genes in different populations. This is especially important in terms of primary health care delivery. Determining the weight of genetic factors in these diseases in which multifactor are effective will determine our approach in the diagnosis of these diseases and in the treatment of patients. Today, the treatment plan according to the person and region has become an increasingly common approach. The distribution of gene mutations often differs significantly between regions. Figures obtained for broad geographic areas may sometimes be far from representative of narrow geographic areas. Therefore, sometimes a mutation may appear unrelated to the disease in one region but may be associated with another. In our study, we tried to examine the difference in the frequency of thrombophilic gene mutations in Erzurum region compared to the general population in our country and other world populations.

Limitations

There are several limitations of our investigation. First, this is a retrospective analysis of prospectively collected data and is therefore subject to the limitations associated with retrospective studies. Second, It is known from which clinic the patient's blood came, but the lack of information about clinical findings, the relationship between thrombophilia and clinical disease and findings could not be examined. Third, this is a single center experience; Therefore, the outcome interpretation is limited by institutional bias. In addition, this study was not randomized. The absence of data on the gender of the patients included in the study is considered as limitation. Moreover, the fact that heterozygous/homozygous differentiation was not made between clinics is also a limitation.

Conflict of Interest

All authors declared no conflict of interest.

Financial Conflict of Interest Disclosure

There is no financial conflict of interest disclosure.

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