BENHA MEDICAL JOURNAL bmj
Print ISSN 1110-208X.
Online ISSN 2357-0016

Original article

Study of Prevalence of Thrombophilic Genes (FVL G1691A, Prothrombin G20210A and MTHFR C677T) Polymorphisms in Patients with Venous Thromboembolism in Benha University Hospital; Cross Sectional Study

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Abstract:

Background: Venous thromboembolism (VTE) is a common and potentially lethal disorder that manifests mainly as deep vein thrombosis (DVT) of the extremities or pulmonary embolism (PE) and occurs because of genetic and environmental risk factors. **Aim of the study:** To evaluate the genetic markers Factor V Leiden (G1691A), Prothrombin gene (PT G20210) and methylene tetra hydro folate reductase (MTHFR C677T) polymorphisms in high-risk patients with venous thromboembolism in Benha University Hospital. Patients and **Methods:** The study consisted of 20 patients of both sexes divided into three groups lower limb DVT group, isolated PE group and DVT complicated by PE group. A venous blood sample collected from patients was used to detect Factor V Leiden (G1691A), Prothrombin gene (G20210A) and methylene tetra hydro folate reductase (MTHFR C677T) polymorphisms by real time polymerase chain reaction (PCR) genotyping. **Results:** We found that the highest genotyping frequency was FVL G1691A polymorphism found in 8 patients (72.7.0%) of included patients with thrombophilic gene polymorphisms, the lowest frequency was F2 G20210A polymorphism found in 2 patients (18.0%) and 5 patients (45%) had MTHFRC677T polymorphism of included patients with thrombophilic gene polymorphisms. FVL G1691A had the highest percentage of 3 patients (25.0%) in lower limb DVT group then MTHFRC677T 2 patients (16.7%) and the lowest percentage was prothrombin G20210A one patient (8.3%). FVL G1691A and MTHFRC677T had an equal percentage in pulmonary thromboembolism group, 2 patients (40.0%) having each polymorphism which is higher than prothrombin G20210A (0.0%) that wasn't detected in this group. FVL G1691A had the highest percentage, 3 patients (100.0) in DVT and pulmonary embolism group while prothrombin G20210A and MTHFRC677T had an equal percentage, one patient (33.3%) having each polymorphism. The genotyping frequency of these polymorphisms had no statistically significant difference between VTE subgroups. **Conclusion:** The present study performed a review of genetic variants associated with venous thromboembolism for understanding the underlying etiology and our results give a strategy of diagnostic evaluations for the individuals at high risk of venous thromboembolism.

Keywords: Venous thromboembolism; Pulmonary embolism; Polymerase chain reaction.

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Received:
Accepted:

Introduction

Pulmonary embolism (PE) and deep vein thrombosis (DVT) are considered to be diverse manifestations of the same disease termed venous thromboembolism (VTE). It is the third most frequent cardiovascular disease. One-third of VTE-related deaths resulted from sudden fatal PE, and undiagnosed PE was found to be the cause of VTE-related deaths in 59% of cases [(1)].

Factor V Leiden (FVL) is the most common and well-studied genetic cause of VTE, followed by the prothrombin G20210A (PTG) gene mutation. These polymorphisms have been proposed as genetically determined.

Procoagulant risk factors for VTE [(2)].

FVL Leiden (G1691A) mutation is caused by the transition of arginine 506 to glutamine which is located in the part of the gene encoding one of the three cleavage sites in factor V (Arg306, Arg506, and Arg679), where activated protein C (APC) inactivates factor Va leading to Factor V hyperactivity which also expresses reduced APC cofactor activity in factor VIIIa inactivation [(3)].

Another important genetic risk factor for VTE is Prothrombin (G20210A), this mutation comprises a guanine to adenine transition at nucleotide 20210 in the 3′-untranslated part of the prothrombin gene (F2) which a gain-of-function mutation where clotting activity is increased by creating more thrombin and fibrin [(4)].

The most common form of genetic hyperhomocysteinemia results from the production of a thermolabile variant of methylene tetrahydrofolate reductase, with reduced enzymatic activity. The gene encoding for this variant contains an alanine to valine substitution at amino acid 677 (677C>T) [(4)].

Increased level of homocysteine (Hcy) in the blood has a toxic effect on the vascular structure [(5)].

The aim of our study is to evaluate the genetic markers (hemostatic and coagulation); Factor V Leiden (G1691A), Prothrombin gene (FII PT G20210) and methylene tetra hydro folate reductase (MTHFR C677T) polymorphisms in high-risk patients with venous thromboembolism in Benha University Hospital.

Patients and Methods

Study groups: - This study was conducted on a total number of 20 patients of both sexes, divided up into three subgroups; lower limb deep venous thrombosis group (12) patients, deep venous thrombosis complicated by pulmonary embolism group (3) patients and isolated pulmonary embolism group (5) patients.

During the period from January 2022 to January 2023, patients with thrombophilia who underwent genetic examination for thromboembolic genes mutations were selected from Department of cardiology, Faculty of Medicine, Benha University Hospital.

The study scheme was approved by the Research Ethical Committee of Benha Faculty of Medicine (MoHP No:0018122017, Certificate No:1017, Study No: Ms.48.12.2021) and informed consent was obtained from the included subjects.

Methods:

Study investigations: -

Laboratory studies include complete blood picture, D-dimer, arterial blood gases (ABG) [(7)]. Radiological studies include venous ultrasound (VUS), Chest radiography Computed tomography pulmonary angiography (CTPA) and Ventilation perfusion (V/Q) scan [(7)].

Genetic analysis:

A molecular study of the thrombophilic genes mutations was

conducted in the Molecular Biology and Biotechnology Unit, Faculty of Medicine, Benha University.

The mutation detection of (FVL G1691A) , prothrombin G20210A and MTHFR C677T genes was performed using real time PCR genotyping.

Sampling:

I. Five ml venous blood sample was obtained from each subject and placed immediately into sterile vacutainer tubes containing ethylene diamine tetra acetate (EDTA) as an anticoagulant.

II. Genomic DNA extraction using PREP-GS Genetics DNA Extraction Kit (DNA-Technology Research & Production, LLC, Russia) [(25)].

III. Measurement of Samples Extracted DNA Concentrations by UV Spectrophotometer measured by Nano drop Spectrophotometer 2000 (Thermo-Fisher Scientific, Wilmington, USA). Readings were taken at wave lengths 260 and 280 nm according to that reported by [(26)] The optical density (OD) ratio at 260 nm and 280 nm was used to estimate the DNA purity. Pure preparations of DNA had OD260/OD280 values of 1.7 - 2.0 respectively.

IV. PCR Amplification using Thrombophilia Susceptibility REAL-TIME PCR Genotyping Kit (DNA-Technology Research &Production, LLC, Russia) [(23)] :

V. The thermocycling program was made using the real-time thermal cycler (Verti thermocycler, Applied Biosystems, Singapore, USA), it included initial denaturation at 94°C for 2 minutes (1 cycle), 35 cycles consisted of denaturation at 94°C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s then final extension at 72°C for 3min (1 cycle).

VI. Molecular study of gene variations of the thrombophilia genes [(22)] :

Allele-specific fluorescent probes were used in the Thrombophilia Susceptibility REAL-TIME PCR Genotyping Kit. For each polymorphism variation, the PCR-mix includes two uniquely labelled allele-specific probes with reporter fluorescent dyes. At each stage, the Real time PCR thermal cycler assessed the fluorescence intensity. The sequence of probes and primers used for detection of thrombophilic gene mutations is shown in table 1. [(36)]

Fluorescence dependence of melting temperature for each tube in the thermoblock was detected and the genotyping frequency of SNPs in thrombophilia genes were determined according to melting temperatures.

Statistical analysis:

Statistical analysis was performed using the Microsoft Office Excel (2021), Statistics Package for Social Sciences (SPSS) and MEDCALC package. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. P-values less than 0.05 were statistically significant [(20)].

Table 1: primer and probe sequences for FVLG1691A, prothrombin G20210A and MTHFRC677T mutations detection.

Genes	**Primer/probe sequences (5' to 3')**		
	Forward primer sequence	Reverse primer sequence	Probe sequence
FVLG1691A	GAA AGG TTA CTT CAA GGA CAA AAT ACC	AGA CAT CGC CTC TGG GCT AAT AG	FAM-ACA GGc gAg GAA T - BHQ1 HEX-ACA GGc aag GAA T - BHQ1
prothrombin G20210A	GCT CCT GGA ACC AAT CCC GTG	CCA GAG AGC TGC CCA TGA ATA G	FAM-CTC AGc gAg CCT C - BHQ1 HEX-CTC AGc aag CCT C - BHQ1

6FAM, 6-carboxy-fluorescein; HEX, hexachloro-fluorescein; BHQ1, Black Hole Quencher 1™.

DOI: 10.21608/bmfj.2024.243200.1926.

Results:

A total of 20 patients suffering from venous thromboembolism were included in this study. The study detected the variants of three genes; FVL G1691A, Prothrombin G20210A and MTHFR C677T. There were 7 males (35.0%) and 13 females (65.0%). The mean age was (43.80), it ranged from 25.0 to 56.0 years.

There were 12 patients (60.0%) had lower limb DVT which is the highest percentage in the studied patients followed by pulmonary thromboembolism group which included 5 patients (25%) then DVT complicated by pulmonary embolism group which included 3 patients (15%). Figure 1 displays percentage of site of VTE in the studied patients.

We found that 11 patients (55%) had thrombophilic gene mutations while the reminder 9 patients (45%) didn't have any of the studied thrombophilic gene mutations.

The highest genotyping frequency was FVL G1691A polymorphism. There were 8 patients (72.7.0%) having FVL G1691A polymorphism in the studied patients with thrombophilic gene mutations;7 patients (63.6%) were heterozygous while one patient (9%) was homozygous. There were 2 patients (18%) of included patients with thrombophilic gene mutations having F2 G20210A polymorphism which is the lowest frequency, all of them were heterozygous carriers and no homozygous carriers were detected, whereas MTHFRC677T was found in 5 patients (45 %), all of them were heterozygous carriers and there were no homozygous carriers.

Table (2) Shows genotyping frequency of thrombophilic gene mutations in the studied patients with thrombophilic gene mutations.

		Studied patients with thrombophilic gene polymorphisms. (N= 11)	
		N	**%**
FVLG1691A	**Heterozygous(GA)**	7	63.6%
	Homozygous (AA)	1	9.0%
F2:G20210A	**Heterozygous(GA)**	2	18.0%
MTHFRC677T	**Heterozygous (CT)**	5	45.0%

FVL G1691A had the highest percentage in lower limb DVT group, found in 3 patients (25.0%), then MTHFRC677T, found in 2 patients (16.7%), and the lowest percentage was prothrombin G20210A, found in one patient (8.3%). FVL G1691A and MTHFRC677T had an equal percentage in pulmonary thromboembolism group, found in 2 patients (40.0%) which is higher than prothrombin G20210A (0.0%) that wasn't detected in this group. FVL G1691A had the highest percentage in DVT and pulmonary embolism group, found in 3 patients (100.0) while prothrombin G20210A and MTHFRC677T had an equal percentage, found in one patient (33.3%).

It was found that genotyping frequency of prothrombin G20210A had no statistically significant difference between VTE subgroups (33.3 % in DVT and pulmonary thromboembolism group, 8.3% in lower limb DVT group and 0.0% in pulmonary thromboembolism group)

(chi square test (X^2) is (2.407) and P-value is 0.300) .

Genotyping frequency of FVL G1691A had no statistically significant difference between VTE subgroups (66.7% heterozygotes and 33.3 % homozygotes in DVT and pulmonary thromboembolism group, 25.0% in lower limb DVT group and 40.0% in pulmonary thromboembolism group) (chi square test (X^2) is (9.155) and P-value is 0.057)

Genotyping frequency of MTHFRC677T had no statistically significant difference between VTE subgroups (33.3 % in DVT and pulmonary thromboembolism group, 16.7% in lower limb DVT group and 40.0% in pulmonary thromboembolism group) (chi square test (X^2) is (1.156) and P-value is 0.561).

Table 3: Genotyping frequencies for three polymorphisms (F2:G20210A, FVLG1691A, and MTHFRC677T) as regard site of VTE .

Items	Site of VTE DVT+ pulmonary embolism		lower limb DVT		pulmonary thromboembolism		Chi- Square test	
	No	%	No	%	No	%	Test value (X^2)	P-value
F2:G20210A								
Heterozygous	1	33.3%	1	8.3%	0	0.0%	2.407	0.300 (NS)
Normal	2	66.7%	11	91.7%	5	100.0%		
FVLG1691A								
Heterozygous	2	66.7%	3	25.0%	2	40.0%	9.155	0.057 (NS)
Homozygous	1	33.3%	0	0.0%	0	0.0%		
Normal	0	0.0%	9	75.0%	3	60.0%		
MTHFRC677T								
Heterozygous	1	33.3%	2	16.7%	2	40.0%	1.156	0.561 (NS)
Normal	2	66.7%	10	83.3%	3	60.0%		

P value> 0.05 is non-significant, P value< 0.05 is significant, X2= Chi- Square test.

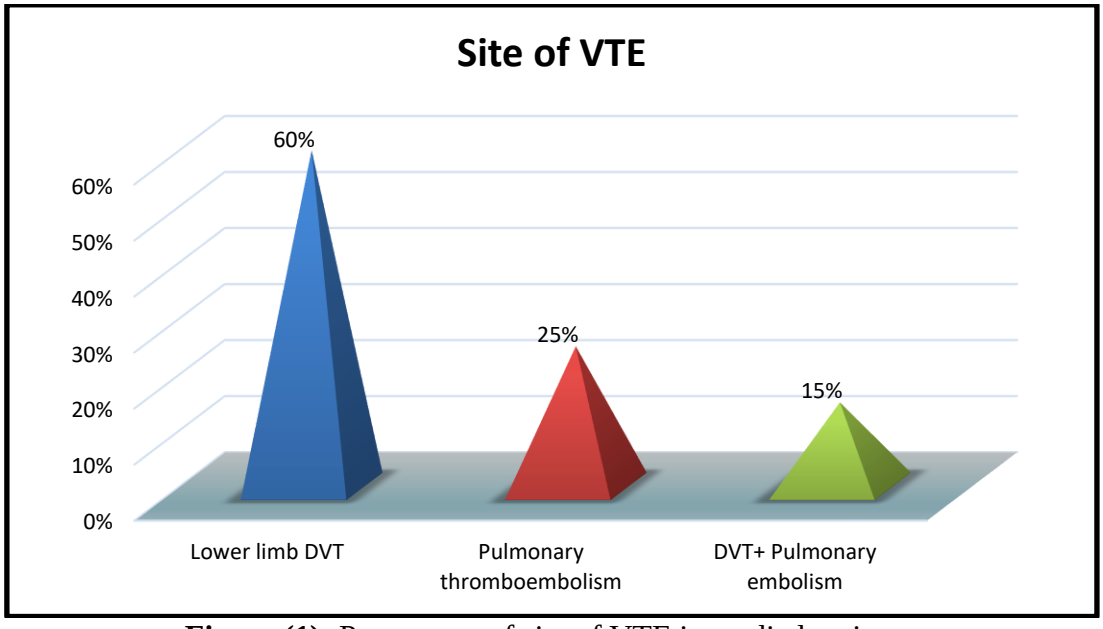

Figure (1): Percentage of site of VTE in studied patients.

DOI: 10.21608/bmfj.2024.243200.1926.

Discussion

Venous Thromboembolism (VTE) is a complex multi-factor disease in which polygenetic factors play a principal role. Synergistic gene-gene and gene-environment interactions contribute to the etiology of VTE and these interactions often lead to hypercoagulability severe enough to result in a disease phenotype [6].

VTE is the third most common cause of vascular mortality worldwide, also the third leading cardiovascular diagnosis after coronary artery disease and stroke. The annual incidence of VTE is 104-183 per 100,000 [6].

Genetic variants in coagulation factors contribute to approximately 50 – 60 % of the variance in VTE incidence. Screening of hereditary thrombophilia factors in VTE patients is crucial for treatment and follow-up planning. Also profiling individual genetic risk could be a useful prevention strategy for VTE [7].

Genetic risk factors for VTE involve gain of function mutations which include factor V Leiden and prothrombin mutation G20210A, which are the most frequent thrombophilic genes polymorphisms observed in the Caucasian population. The prevalence reaches 5% for FV Leiden mutation and 2% for G20210A mutation. In contrast, they are much rarer in African and Asian populations [8, 9].

The aim of our study is to evaluate the genetic markers (hemostatic and coagulation) ; Factor V Leiden (G1691A), Prothrombin gene (FII PT G20210) and methylene tetra hydro folate reductase (MTHFR C677T) polymorphisms in high-risk patients with venous thromboembolism in Benha University Hospital.

We found that the percentage of FVLG1691A polymorphism among the studied VTE patients was 40.0%, there were (35.0%) heterozygous carriers and (5.0%) homozygous carriers.

A study conducted by [29] found a similar percentage of FV Leiden in which its prevalence was 20%-35% of the total patients presenting with VTE.

Similarly, a study [10] reported that the FVL prevalence was approximately 20% in unselected VTE patients, heterozygous mutations of FVL being observed in 12–20% of patients with incidental VTE. Homozygous FVL mutations are less frequent, with an incidence rate of 0.02 % [10].

Our results were also like an Iranian study conducted where they found that 35.8% of the total patients presenting with VTE were positive for FVL mutation with 26.9% being heterozygous and 8.9% as homozygous [11].

A study conducted before found that among the patients with thrombotic events history, 21.5% were carriers of FVL mutation in heterozygosity [8].

This can be explained by the prevalence of FVL, one of the frequently observed and important risk factors for genetic thrombophilia, varies in different populations due to ethnicity and geographic differences. It varies from 0 to 15% according to ethnicity and geographic distribution worldwide [8].

This present study found that genotyping frequency of FVL G1691A polymorphism had no statistically significant difference between VTE subgroups (chi square test (X^2) is (9.155) and P-value is 0.057)

In contrast to our results, a meta-analysis conducted previously reported that the prevalence of Factor V Leiden to be significantly higher in patients with isolated DVT than in patients with pulmonary embolism (with or without DVT) [24].

We found that the percentage of F2 G20210A polymorphism was 10.0% in the studied VTE patients, all of them were heterozygous and there were no homozygous carriers.

Our results agreed with others who found that heterozygote genotype of FII 20210 is present in 6–18% of patients with VTE [27].

Moreover, it was found that the prevalence of prothrombin G20210A mutation has

been reported to range from 6–16% in patients with VTE [28]. Also, others [13] demonstrated that heterogeneous variants for prothrombin G20210A mutations are more common than homogeneous variants, this is in accordance with our results.

It was found that the prothrombin G20210A mutation has an incidence that varies from 1% to 6% in general Caucasians and from 5% to 19% in patients with VTE while it is very rare in individuals from Asia and African countries [12].

All these differences in the results are attributable to the geographical diversity in the genetic distribution pattern of the disorder [12].

We found that genotyping frequency of F2 G20210A polymorphism had no statistically significant difference between VTE subgroups (Chi- Square test (X2) equals 2.407 and P-value equals 0.300).

On the other hand, some researchers found that among individuals with DVT, 20210G>A heterozygotes had a significantly higher rate of PE (32%) than those with the factor V Leiden variant (19%) or those without thrombophilia (17%). 20210G>A heterozygotes are also at increased risk of developing isolated PE [15].

In our study, we found that there were no homozygote genotypes for prothrombin G20210A mutation while there were 5.0% with homozygote genotypes for FVL mutation in the studied patients.

Our data were similar to the study done in 2019 [13] which found that homozygosity for prothrombin G20210A mutation is rarer than homozygosity for the FVL 1691G>A variant. However, the risk for VTE is high and has been reported to be 30 times increased.

We found that the percentage of MTHFR C677T polymorphism was 25.0% among studied VTE patients, all of them were heterozygous while there were no homozygous carriers.

This is consistent with a large scale meta-analysis conducted by a group of researchers [31] which showed that there was no evidence for an association with homozygotes for the MTHFR C677T variant and VTE also, no association between the C677T polymorphism and venous thrombosis as reported by [32] this can be explained by an MTHFR polymorphism increases serum homocysteine in low folate states, this renders this genetic marker less relevant for an increased risk of VTE [33] and also, studies cast doubt on the relationship between the presence of MTHFR mutations or homocysteine and vascular disease[33,34]. high levels of homocysteine are not independently causative for vascular disease [35].

On the other hand, previously published data of similar studies which found that heterozygote genotype for MTHFR C677T was present in (40.0%) while homozygote genotype were present in (7.5%) of studied VTE patients [14-16].

We found that genotyping frequency of MTHFR C677T polymorphism had no statistically significant difference between VTE subgroups (Chi- Square test (X2) equals 1.156 and P-value equals 0.561), this finding was in agreement with others [17-19]

We found that sixty percent of patients in our study had lower-extremity DVT, whereas twenty-five percent had pulmonary thromboembolism, and fifteen percent had both.

Similarly, [26] found that approximately 2/3 of VTE is clinically manifested as DVT, and 1/3 manifests as isolated PE or PE coexisting with DVT.

Also, [25] found that the most common venous thromboembolic disease is DVT. Without PE, the annual incidence of DVT is 45-117 per 100,000 people and the incidence of PE is 29-78 per 100,000 people.PE develops in approximately 20% of untreated DVTs and progresses fatally with a rate of 10-20%.

DOI: 10.21608/bmfj.2024.243200.1926

Conclusions

FVLG1691A polymorphism is most prevalent among high-risk venous thromboembolic patients followed by prothrombin G20210A.All young patients presenting with unprovoked or recurrent VTE need to be screened for heritable thrombophilia. This can aid in deciding the duration of anticoagulant therapy in different clinical settings, planning for follow up according to the results, planning for prophylactic anticoagulation in high-risk situations and prediction of the prognosis. Further studies are needed to assess the importance of genetically determined thrombophilia for the risk stratification of patients with VTE and planning the duration of anticoagulant treatment, also further studies are needed to assess the prevalence of the studied thrombophilic gene mutations in a large-scale cohort.

Conflict of interest

None of the contributors declared any conflict of interest.

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To cite this article: Amal Idris, Sania K. Elwia, Yasmin M. Marei, Ahmed M. Bendary, Moamena Y. Mahmoud. Study of Prevalence of Thrombophilic Genes (FVL G1691A, Prothrombin G20210A and MTHFR C677T) Polymorphisms in Patients with Venous Thromboembolism in Benha University Hospital; Cross Sectional Study. BMFJ XXX, DOI: 10.21608/bmfj.2024.243200.1926.