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Genetic mutations among a group of patients with unstimulated thrombosis in Sulaymaniyah Northeastern Iraq

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Abstract:

BACKGROUND: Thromboembolism is a complex disease caused by different acquired and inherited factors. The common mutations including Factor V leiden (FVL), prothrombin (PTG), and methylenetetrahydrofolate reductase (MTHFR) are important inherited causes in both venous and arterial thrombosis.

OBJECTIVES: The aim of this study was to determine the frequency of the common three thrombophilia mutations in a group of patients with unstimulated thrombosis in comparison to healthy controls.

PATIENTS AND METHODS: This is a prospective case-control study of mutations in 100 samples, 50 patients with documented thrombosis referred to the Sulaymaniyah Public Health Laboratory for thrombophilia screening, as well as other 50 healthy age-matched controls. Multiplex polymerase chain reaction and reverse hybridization to oligonucleotide-specific probes, was used to detect FVL G1691A, PTG20210A, and MTHFRC677T mutations. Assays for other thrombophilia markers (Protein C, Protein S, and Antithrombin) were also performed.

RESULTS: FVL was found in 22% of patients versus 6% of controls and associated with a 4-fold increased risk of thrombosis OR: 4.41, $P = 0.021$. MTHFR and PTG were found in 42% and 4%, respectively among patients with no significant increased risk. Mutations with more than one thrombophilia markers further increased the risk of thrombosis to 5-fold $P = 0.025$. Deep-vein thrombosis was the most common form of thrombosis and it is more in a young age group.

CONCLUSIONS: FVL is significantly related to the risk of thrombosis development and presence of other thrombophilia markers further increase thrombotic risk, so understanding these facts may encourage screening those patients and may help proper management.

Keywords:

Factor V leiden, methylenetetrahydrofolate reductase, polymorphisms, thrombophilia

Introduction

Thromboembolism (TE) results from either acquired or inherited factors, it can occur inside the vein or artery.[1,2] the most common hereditary determinants are mutations in factor V (FV), prothrombin (PTG), and methylenetetrahydrofolate reductase (MTHFR) C677T.[3-5] Resistance to activated protein C (APC), in the majority of cases, after effects of missense mutations actuated by the substitution of guanine by adenine at the position of nucleotide 1691 located in exon 10 of the FV gene (G1691A), known as FV Leiden (FVL). This transformation makes the substitution of arginine by glutamine at amino acid 506, situated at one of the sites where FV is recognized, cleaved, and inactivated by the APC. As a result, FVa.

is not sufficiently inactivated, and thus predisposes thrombosis.[3,6] The substitution of guanine by adenine at nucleotide 20210 in the 3′ untranslated locale of the PTG

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gene (G20210A), increases the messenger RNA stability, bringing about raised PTG plasma levels.[7,8] The role of deficiency of natural anticoagulants; antithrombin (AT), protein C (PC), and protein S (PS) has been settled in thrombophilia.[9-12] The frequency of these hereditary mutations has been designated by numerous authors,[13,14] however as far as we know, a few reports have been done in our locality.

The study aimed to define the frequency of these common three thrombophilia mutations in a group of patients with unstimulated thrombosis in comparison to healthy controls.

Patients and Methods

The design of this work is prospective case-control study in which a consecutive eligible newly diagnosed patients with documented thrombosis (using ultrasonography, color Doppler, venography/high resolution CT studies) attending the Sulaymaniyah public health laboratory, main governmental laboratory in the city, for thrombophilia screening were enrolled in this study through 6 months from the beginning of December 2017 to the end of May 2018.

We excluded patients with acquired causes such as recent history of trauma or surgery (<3 months), smoking, prolonged bed rest, recurrent deep-vein thrombosis (DVT) pregnancy, patients on oral contraceptives, patients on oral anticoagulants, patients with positive markers for lupus anticoagulant, and patients with liver disorders. Consenting healthy ages matched unrelated volunteers and from the same region, referred to in the same study period, were enrolled as a control group. They had no history of thrombosis, not smoker, were not suffering from any illness of significance, and not taken any drugs at the time of sampling.

The approval of the local Ethical Committee was obtained at the beginning of the study, and proper informed consent from both of the patients and control was also claimed. The recommendations and rules of the Helsinki Declaration were followed throughout the conduction of the study. Both EDTA and citrated blood samples were retrieved from the patients and the control group. EDTA samples were deeply frozen until the time of DNA extraction (usually within 1 week from sample collection), the citrated samples were centrifuged within 1 h and plasma extracted and deeply frozen for PC, PS, and AT analysis.

Peripheral blood leukocytes are the source of DNA extraction (according to FV-PTH-MTHFR strip Assay, Vienna Lab, Austria protocol [http://www.viennalab.com]), then the process is followed by polymerase chain reaction (PCR) amplification through PCR (30 cycles): 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, and oligo-specific probes were used for reverse hybridization. The FV-PTH-MTHFR Strip Assay kit gives reagents to *in vitro* amplification utilizing biotinylated primers, then the hybridization process of amplification products to a test strip that contains oligonucleotide probes for the targeted allele which is then immobilized as an array of parallel lines. From that point, bound biotinylated sequences are recognized utilizing both color substrates and streptavidin-alkaline phosphatase. In this study, we used the Eppendorf (Germany VAPO protect), Mastercycler ® pro (USA). This assay procedure covers three mutations: FV G1691A (Leiden), PTG20210A, and MTHFRC677T. We analyze for other proteins (PC, PS, and AT) through STA COMPACT fully automated coagulation analyzer (Diagnostica Stago 9/France).

Statistical analysis

SPSS version 25.0 (Armonk, NY: IBM Corp, USA) for Windows was used for data analysis. Means and SD were calculated in continuous data, and the Independent *t*-test was used for differences. The frequency proportion for categorical data and Chi-square with an odds ratio (OR) provided with a 95% confidence interval (CI) was computed to test the risk between different mutations and thrombosis. *P* value was considered statistically significant at a level of <0.05.

Results

Out of all cases admitted to our laboratory for thrombophilia testing during the period of the study, only 50 eligible consenting patients, according to our criteria mentioned earlier, were analyzed for thrombophilia markers they were proven to suffer from different venous or arterial thrombosis. The control group was also 50 ages matched healthy persons.

There were 32 female and 18 male patients with a mean age of 34 years (18–49 years); 31 and 19 patients were aged ≤40 and >40 years, respectively. The sex ratio in the control group was more balanced (21 females vs. 29 males). The thrombophilia mutations, sites, and demographic parameters in cases and controls are shown in Table 1. Most of cases were of DVT (n = 22), followed by abortion (n = 11) and ischemic stroke (n = 10). Eleven carriers of FVL were found in the patient group as opposed to only 3 in the control group, whereas 21 for MTHFR and two for PTG20210A compared to 15 and one in the control group, respectively. The deficiency of PS (n = 5) was found only among the cases group, whereas no AT or PC could be found in any group.

The distributions of mutation frequency in cases and controls group with their risks in the causation of thrombosis are described in Table 2. This result showing that only FVL among the other three polymorphisms was associated, on its own, with a significantly increased risk of thrombosis.

The detail of mutation and allele frequency is shown in Table 3. FVL (G> A) was seen in homozygous state (AA) in 5 patients forming 10% of cases, heterozygous state (GA) seen in 6 patients (12%). Three persons of the control group were heterozygous. A allele frequency (patients/controls) was around 4% and this imparts a significant increase in thrombosis with OR of 4.41 (95 % CI: 1.15–16.96). PTG 20210 (G> A), on the other hand, was seen in the heterozygous state in 4% of patients and 2% of controls. A allele frequencies were 2% and 1% respectively and thus confer an increased risk of thrombosis to 2 fold, though it was of no statistical significance $P = 0.561$. There was no homozygous state for PTG in both groups. MTHFR C677 T was detected in 30% of patients and 42% of the controls, 21 patients were of heterozygous state (CT), while there were three persons among control group in homozygous state (TT) and three persons of heterozygous state (T allele frequencies of 1.4%), which results in mild increase in risk of thrombosis.

Table 1: Thrombophilia mutations, sites, and demographic parameters in cases and controls (*n*=50)

	Controls	Cases
Age (years), mean (range)	37 (25-49)	34 (18-49)
Gender		
Male	29 (58)	18 (36)
Female	21 (42)	32 (64)
Thrombosis		
Ischemic stroke	-	10 (20)
Portal vein thrombosis	-	4 (8)
DVT	-	22 (44)
Pulmonary embolism	-	2 (4)
Abortion	-	11 (22)
MI	-	1 (2)
Thrombophilia markers		
FVL total	3 (6)	11 (22)
MTHFR total	15 (30)	21 (42)
PTG20210A total	1 (2)	2 (4)
PC deficiency	0	0
AT deficiency	0	0
PS deficiency	0	5 (10)

DVT=Deep-vein thrombosis, FVL=Factor V Leiden, MTHFR=Methylenetetrahydrofolate reductase, PTG=Prothrombin, PC=Protein C, AT=Antithrombin, PS=Protein S, MI=Myocardial infarction

Table 2: Distribution of mutation frequency in cases and controls group with their risks

Mutations	Controls (%)	Cases (%)	OR (95% CI)	*P*
FVL	3 (6)	11 (22)	4.41 (1.15-16.96)	0.021
MTHFR	15 (30)	21 (42)	1.69 (0.74-3.85)	0.211
PTG20210A total	1 (2)	2 (4)	2.04 (0.17-23.26)	0.560
Total	19 (38)	34 (68)	2.45 (1.09-5.49)	0.028

OR=Odds ratio, CI=Confidence interval, FVL=Factor V Leiden, MTHFR=Methylenetetrahydrofolate reductase, PTG=Prothrombin

The presence of thrombophilia markers in the whole sample with relation to thrombosis is depicted in Table 4. Positivity for at least one marker of thrombophilia among cases ($n = 29$), was higher than the controls ($n = 17$) with an OR of 2.68 ($P = 0.016$).

Odds of TE were also increased in cases that had positivity of more than one thrombophilia mutation (OR = 2.66, 95% CI: 0.49-14.44, $P = 0.240$). The presence of thrombophilia markers and mutations was present in 9 cases and 2 of controls leading to an OR of 5.26, 95% CI: 1.07–25.77, $P = 0.025$.

Discussion

The Sulaymaniyah region (where the study is conducted) is located in northeastern Iraq spans around 20,000 km^2 with a population of more than two million mainly of Kurdish ethnicity.

TE was proven to be an important cause of morbidity and mortality in all age groups notably in adulthood.[15] The interaction between genetic and environmental factors is playing a role in the causation of it,[16] and hence, we included in our study only the unstimulated cases of thrombosis. Very few reports on different thrombophilic mutations, especially in thrombotic cases have been done in our locality.

We studied thrombophilia mutations in 100 samples (50 cases and 50 healthy controls), it was reported in 29 patient (58%) and 17 in control group (34%) (including five combined mutations for patients and two for

Table 3: Thrombophilia mutations and allele frequency in cases and controls group (*n*=50)

Mutations	Cases group		Controls group	
	Homozygous, *n* (%)	Heterozygous, *n* (%)	Homozygous, *n* (%)	Heterozygous, *n* (%)
FVL G1691A	AA: 5 (10)	GA: 6 (12)	AA: 0	GA: 3 (6)
MTHFR C677T	TT: 0	CT: 21 (42)	TT: 3 (6)	CT: 12 (24)
PTG20210A total	AA: 0	GA: 2 (4)	AA: 0	GA: 1 (2)
Total	5 (10)	29 (58)	3 (6)	16 (32)

FVL=Factor V Leiden, MTHFR=Methylenetetrahydrofolate reductase, PTG=Prothrombin

Table 4: The presence of thrombophilia markers in the whole sample in relation to thrombosis

	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)	*P*
Without mutations	33 (66)	21 (42)	-	Reference
With mutation	17 (34)	29 (58)	2.68 (1.19-6.03)	0.016
More than one mutation	2 (4)	5 (10)	2.66 (0.49-14.44)	0.240
Mutations and other markers	2 (4)	9 (18)	5.26 (1.07-25.77)	0.025

OR=Odds ratio, CI=Confidence interval

controls) which forms a statistically significant difference $P = 0.028$ and this confers an OR of risk to 2.45 (95% CI: 1.09–5.49), this risk is further increased if more than one mutation is present though it is of no statistical significance, this is also have been reported in many studies.[17]

The FVL mutation was founded in 22% ($n = 11$) of TE cases (5 homozygous and 6 heterozygous) in comparison to 6% of control (none of them whereof homozygous form) this figure is much lower than that reported in the Western countries (64%),[14] but is more than that in Indian TE subjects (12%–12.5%).[18] In Iraq, other studies on the frequency of FVL (among healthy controls) were reported as 1.25% in Dohuk.[19] and 3% in Baghdad,[20] in our healthy controls, it was 6%. In another study conducted on DVT patients, it was 16%.[21]

For the neighboring countries, the figures in patients were as follows: 23% in Jordan,[22] 21% in Turkey,[23] 15% in Kuwait,[24] 11.4% in Iran,[25] no data from Saudi Arabia and Syria. This variation could be explained by different ethnic backgrounds.

In our study, FVL was associated with more than four times increased risk of thrombosis in cases, OR 4.41 (95% CI: 1.15–16.96) $P = 0.021$, this aligns with many studies.[26-28]

MTHFR C677T mutation was the most common in our cases 21 (42%) in both alleles than the control group 15 (30%), it has been demonstrated that elevated levels of homocysteine associated with MTHFR have been found to confer an increased risk of TE (2–4 times the normal person's risk),[29-31] but isolated mutation is not a cause for thrombosis except if being related with some other hereditary or acquired risk factor as exhibited in this study just as in other related studies.[32] Some studies uncover that this affiliation is disputable.[33] In our sample, it was associated with increased risk, but it is of no statistical significance ($P = 0.211$) which is similar to a recent study done by Şenol and Kargün,[34] the presence of homozygous allele (TT) in our control group confirms that it is not significant for thrombosis developments, though this association has been reported by Jang *et al*.[35]

In our study, PTG mutation was double in patients than control 2:1 with no homozygous allele (AA) and was found to impart risk factor in TE but is not statistically significant, similar to other meta-analysis study done by Joaquín *et al*.,[36] and other studies,[37] which describe a positive association of PTG with cerebral vein thrombosis especially if AA genotype is inherited.

The risk of having TE in our sample is 5-fold increased in those with additional thrombophilia markers, in our cases, we have 5 (10%) patients with PS deficiency (OR = 5.26, 95% CI: 1.07–25.77 $P = 0.025$). Studies in Indian TE patients have also reported a comparable prevalence of PS deficiency as 6.5%–19.0%.[38] In the Caucasian population, it is around 5%.[13] No deficiency of PC or AT was reported in our sample.

Regarding age group many studies revealed the association of thrombosis to the early age group <40 years,[39,40] in our study; the young age (<40 years old) group was more than those with older age group but with no statistically significant difference $P = 0.084$, this is explained by the inherited nature of thrombosis which is usually occurring in young patients with unstimulated causes for thrombosis as a direct effect of mutations.

Some limitations of our study include the small number of patients and controls, so large scale study may help to overcome the low frequency of prothrombotic carriers among participants and in many instances, it was not feasible to apply thrombophilia testing in our control group.

Conclusions

Our data show that FVL is significantly related to the risk of TE development and that the addition of other thrombophilia markers increases the thrombotic risk, understanding of these facts may encourage screening in patients and may help proper management.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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