Science

Thrombophilic Mutations in Iranian Patients With Thrombophilia

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ABSTRACT

Objectives: To investigate the frequency and type of Factor V Leiden (FVL), prothrombin, and methylenetetrahydrofolate reductase (MTHFR) mutations in ethnic Iranian patients and to evaluate the correlation between platelet count and thrombotic mutations.

Methods: This descriptive epidemiologic case study was performed with 208 patients with thrombotic disorders and hemorrhaging who were referred to Payvand Medical and Specialty Laboratory in Tehran, Iran, from 2007 through 2010. Peripheral blood samples from patients with abnormal thrombosis were analyzed for FVL, prothrombin G20210A, and MTHFR (A1298C and C677T) mutations, respectively.

Mutation analysis was accomplished by polymerase chain reaction (PCR)–reverse dot blot. Platelet counts were obtained by automated cell count.

Results: Thrombophilic mutations were observed in 105 cases of 208 patients, including 13 patients with FVL, 51 patients with MTHFR C677T mutation, 33 patients with MTHFR A1298C mutation, and 8 patients with prothrombin G20210A mutation. Eighteen patients showed 2 simultaneous mutations and 3 patients showed 3 simultaneous mutations. According to our results, platelet counts were in normal range, so no statistically significant difference was observed between platelet count and mutation type.

Conclusions: The frequency of MTHFR C677T mutation is higher than that of FVL and/or prothrombin 20210A mutations in ethnic Iranian patients with thrombophilia. Also, thrombophilic mutations were not observed to have any effect on platelet count in patients with thrombophilia. Regarding the high prevalence of MTHFR C677T mutation in our region, family members of any patient with the MTHFR C677T mutation should be screened to identify this genetic mutation.

Keywords: Factor V Leiden, prothrombin 20210A, MTHFR C677T, A1298C, thrombophilia, Iranian

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Abbreviations

FVL, Factor V Leiden; APC, activated protein C; MTHFR, methylenetetrahydrofolate reductase; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; SSOP, sequence-specific oligonucleotide probes; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; PT, partial thrombosis; PLT, platelet; RFLP, restriction fragment length polymorphism; BP, base pair

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Factor V Leiden (FVL) is caused by a single point mutation at nucleotide 1691, leading to the replacement of arginine at position 506 in the activated protein C (APC) cleavage site of factor Va by glutamine. This mutation occurs in approximately 5% of the white population.[1] The single base substitution (G>A) at nucleotide 20210 in the 3'-untranslated region of the prothrombin gene (11p11.2) is associated with approximately a 25% increase in plasma thrombin activity and a 2.7-fold increased risk for venous thrombosis.[2] The 2 mutations of the methylenetetrahydrofolate reductase (MTHFR) gene (1p36.3) that are most defined in the literature are a missense mutation that substitutes cytosine with thymine at nucleotide 677, which results in the conversion of alanine to valine, and the transversion of adenosine to cytosine at nucleotide 1298, which leads to the conversion of glutamate to alanine.[3,4] The influence of these mutations varies from a mild

to severe decrease in MTHFR enzyme activity. FVL and prothrombin G20210A substitution, which both exhibit an autosomal dominant inheritance pattern, are the most important thrombotic factors in humans.[2,5,6] FVL is reported in 50% of familial thrombosis in the European population.[7] Significant correlation between FVL and sickle cell disease in Iranian population has been reported.[8] Another study on 434 ethnic Iranian subjects has confirmed 7 cases of heterozygous mutation in prothrombin G20210A and no mutations were homozygous.[9] However, deficiency in vitamins B_6, B_{12}, and folic acid may potentially lead to decrease in MTHFR activity.[4,10] Two distinguished mutations in this gene including C677T (Ala222Val) and A1298C (Glu429Ala) would decrease its activity. The thrombophilic role of MTHFR mutations is less than those of FVL and prothrombin G20210A substitution, which means that MTHFR gene mutations could slightly increase the risk of arterial or venous thrombosis.[7] More specifically, FVL mutation, prothrombin G20210A, and MTHFR C677T increase the risk of myocardial infarction and ischemic stroke in female patients less than 55 years ol).[11]

The goal of the present study is to determine the prevalence of FVL, prothrombin G20210A, MTHFR A1298C, and C677T mutations among ethnic Iranian patients with a history of thrombotic disorders. Also, we explore the correlation between platelet count and thrombotic mutations.

Methods

Study Design and Patients

Our descriptive epidemiologic case study was conducted with 208 patients with thrombotic disorders and hemorrhaging who were referred to Payvand Medical and Specialty Laboratory in Tehran, Iran, from 2007 through 2010. The study was approved by the Ethics Committee of the Tehran Medical Branch of Islamic Azad University.

Inclusion Criteria

The referred patients had clinical complications, such as venous and arterial thrombosis, hemostatic problems, seizures, stroke, abdominal tumors, and ischemia. They were evaluated for thromboembolism by standard imaging methods. Eventually, patients with thrombotic disorders and hemorrhaging were included in this study.

Blood Sampling and Detection of Mutations by PCR-reverse Dot Blot

In this research, peripheral blood samples of the patients with abnormal thrombosis were analyzed for mutations in Factor V Leiden, prothrombin G20210A, and MTHFR A1298C and C677T. The blood samples were collected in vacuum tubes containing K2 ethylenediaminetetraacetic acid (EDTA) anticoagulant. After proper mixture by standard methods, the samples were centrifuged for 5 minutes at 2500*g* to separate the buffy coat layer. Genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit (F. Hoffman-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions. The quality of extracted DNA was analyzed by agarose gel electrophoresis. DNA concentration and purity were evaluated by the absorbance spectroscopy at 260 nm and calculation of the absorbance ratio at 260 to 280 nm.

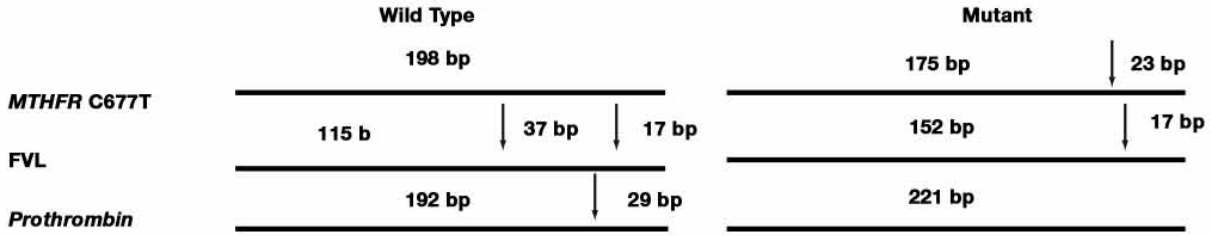
PCR-reverse dot blot was performed according to previously published methods.[12] Polymerase chain reaction (PCR) was performed using FVL, prothrombin, and MTHFR kits (Tib Molbiol GmbH, Berlin, Germany). The PCR reaction mixture contained 35 µl of master mixture provided in the kit, 5 µl 10× polymerase buffer, 5 µl of $MgCl_2$ solution (final concentration of 2.5 mM $MgCl_2$), 1 unit of thermostable DNA polymerase, and a 150 to 300 ng sample of DNA. Finally, distilled, autoclaved water was added, to obtain a volume of 50 µl (**Figure 1**).

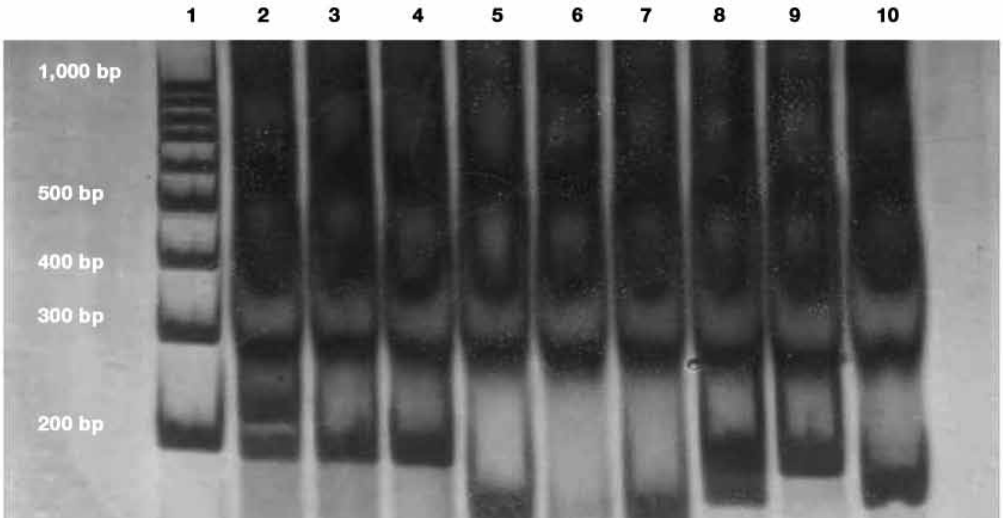
In the reaction, fragments of the FVL and prothrombin genes were amplified, as well as 2 fragments of the MTHFR gene, with specific, biotin-labeled primers by multiplex amplification. The amplified gene fragments were characterized in a hybridization reaction with sequence-specific oligonucleotide probes (SSOP) for wild-type and mutant alleles, which were immobilized on nitrocellulose (ie, reverse hybridization). During hybridization, the denatured amplified DNA binds to the gene probe attached to the strips (**Figure 2**). A highly specific washing procedure ensures that the hybrids only survive if the probe's sequence is 100% complementary to that of the amplified DNA. Streptavidin-coupled alkaline phosphatase binds to the hybrids of the gene probe and biotin-labeled amplified DNA. This complex will then be detected by a color reaction of 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) alkaline phosphatase substrate solution.

In hybridization testing, normal and mutant amplicons are hybridized with wild-type and mutant allele-specific

Figure 1

The polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) results for all 4 detected mutations in the study population. Lane 1: 100 base pair (bp) DNA ladder. Lane 2: Prothrombin G20210A heterozygotic allele. Lane 3: Prothrombin G20210A wild-type allele. Lane 4: Patient DNA (wild-type allele). Lane 5: Factor V Leiden (FVL) heterozygotic allele. Lane 6: FVL wild-type allele. Lane 7: Patient DNA (heterozygotic allele). Lane 8: Methylenetetrahydrofolate reductase (MTHFR) C677T heterozygotic allele. Lane 9: MTHFR C677T wild-type allele. Lane 10: Patient DNA (homozygotic allele). Accession number in GenBank: MTHFR(AY338232),FV (NM_000130), PT (M17262). Accession number in GenBank: MTHFR(AY338232),FV (NM_000130), PT (M17262).





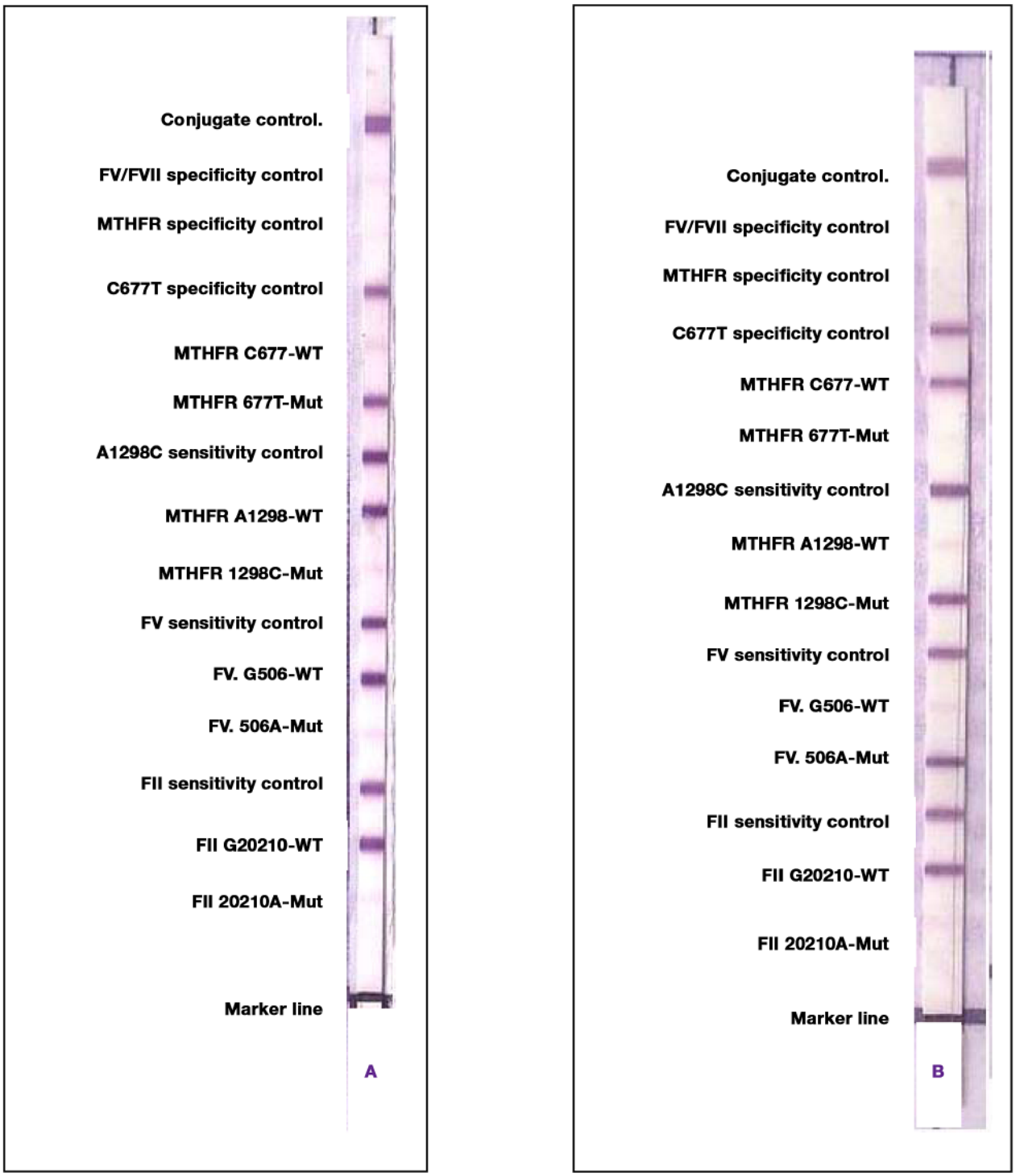
oligonucleotide probes, respectively. Positive internal control for each polymorphism indicates the correct function of PCR amplification and hybridization. The internal control should be positive for each polymorphism; otherwise, the results will not be reliable. In healthy patients, internal control and wild-type bands were positive. In the heterozygous form, the mutant band will appear beside the normal one and in the homozygous form, only the mutant band will appear beside the internal control band.

Statistical Analysis

The obtained data was analyzed using SPSS software, version 15.0 (SPSS Inc, Chicago, IL). The Chi^2 test and Spearman correlation were used for analysis of the relationship between gene mutations. *P* values of less than .05 were considered statistically significant.

Figure 2

PCR products attachment to the genes probes. **A**, patient with homozygous MTHFR 677T mutation; **B**, patient with simultaneously homozygous MTHFRA1298C and FV 506A mutations.FVL-FII indicates, Factor V Leiden- Factor II ; MTHFR, methylenetetrahydrofolate reductase; WT, wild type; and Mut, mutation.



Results

A total of 92 male and 116 female patients with suspected thrombophilia were studied for thrombophilic mutations, with a mean (SD) age of 33.3 (18.27) years (range, 1-82) and 30.3 (16.92) years (range, 1-71) for male and female patients, respectively. Among the 208 patients, thrombophilic mutations were observed in 105 cases; 103 cases showed no thrombophilic mutations despite their thrombosis. **Table 1** shows the number of patients who tested homozygous and heterozygous for each thrombophilic mutation.

Factor V Leiden

Thirteen patients tested positive for FVL G506A mutation; one case (0.5%) was homozygous, and 12 cases (5.8%) were heterozygous. FVL mutation rates in females and males were 2.6% and 10.9%, respectively. The percentage of heterozygous mutation in males was 10.9% and in females was 2.6% (**Table 2**). A significant difference was observed in the prevalence of FVL mutation between males and females (P = .02).

MTHFR C677T Mutation

Fifty-one patients tested positive for the MTHFR C677T mutation, of which 43 patients (20.7%) were heterozygotic and 8 patients (3.8%) were homozygotic. The percentages of MTHFR C677T mutation in males and females were 21.8% and 26.8%, respectively (**Table 2**); this difference was not statistically significant (P = .48).

MTHFR A1298C Mutation

Thirty-three patients tested positive for the MTHFR A1298C mutation, of which 26 patients (12.5%) were heterozygotic

Table 1. Homozygous and Heterozygous Patients With Each Type of Thrombotic Mutation[a]

Mutation Type	A1298C, No. (%)	C677T, No. (%)	PT, No. (%)	FVL, No. (%)	Total
Heterozygous	26 (24.8)	43 (41.0)	8 (7.6)	12 (11.4)	**89 (84.8)**
Homozygous	7 (6.7)	8 (7.6)	0	1 (1.0)	**16 (15.2)**
Total	**33 (31.4)**	**51 (48.6)**	**8 (7.6)**	**13 (12.4)**	**105 (100)**

Abbreviations: PT, partial thrombosis; FVL, Factor V Leiden.
Accession number in GenBank: MTHFR(AY338232),FV (NM_000130), PT (M17262).

Table 2. Homozygous and Heterozygous Mutations of Each Gene[a]

		Sex, No. (%)		
	Variable	Male	Female	Total, No. (%)[b]
MTHFR A1298C	Normal	81 (38.9)	94 (45.2)	175 (84.1)
	Heterozygotic	9 (43.3)	17 (8.2)	26 (12.5)
	Homozygotic	2 (1.0)	5 (2.4)	7 (3.4)
Prothrombin G20210A[c]	Normal	88 (42.3)	112 (53.8)	200 (96.2)
	Heterozygotic	4 (19.2)	4 (19.2)	8 (3.8)
MTHFR C677T	Normal	72 (34.6)	85 (40.9)	157 (75.5)
	Heterozygotic	18 (8.7)	25 (12.0)	43 (20.7)
	Homozygotic	2 (1.0)	6 (2.9)	8 (3.8)
FVL G506A	Normal	82 (39.4)	113 (54.3)	195 (93.8)
	Heterozygotic	9 (4.3)	3 (1.4)	12 (5.8)
	Homozygotic	1 (0.5)	0	1 (0.5)

Abbreviations: FVL, Factor V Leiden; MTHFR, methylenetetrahydrofolate reductase.
[a] *N = 208.*
[b] *Percentages may not sum evenly because of rounding.*
[c] MTHFR *gene: OMIM 607093;* prothrombin *gene: OMIM 176930 for coagulation factor II or OMIM 188050 for thrombophilia due to thrombin defect.*
Accession number in GenBank: MTHFR(AY338232),FV (NM_000130), PT (M17262).

and 7 patients (3.4%) were homozygotic. Mutation rates in males and females were 12% and 19%, respectively; 10.9% of males and 10.6% of females were heterozygotic (**Table 2**). According to our findings, there were no significant differences in the prevalence of MTHFR A1298C mutation between males and females (P = .42).

Prothrombin G20210A Mutation

Only 8 patients (3.8%) demonstrated the prothrombin G20210A heterozygous mutation, of which 4.3% were observed in males and 3.4% in females (**Table 2**). Despite the mutation rate being slightly higher in males, no significant difference was observed in the prevalence of prothrombin G20210A mutation between males and females (P = .74)

Simultaneous Occurrence of Mutations

Sixty patients had only 1 mutation, of whom 28 (46.7%) were male and 32 (53.3%) were female. The mean (SD) age of these patients was 33.4 (19.9) years. Mutation types in these patients included 16 (26.7%) MTHFR A1298C, 32 (53.3%) MTHFR C677T, 3 (5%) prothrombin G20210A, and 9 (15%) FVL.

Eighteen patients had 2 simultaneous mutations. The mean age of these patients was 33.8 (14.56) years; the 8 males had a mean age of 28.8 years and the 10 females had a mean age of 37.4 years. Twelve of them showed heterozygous mutation for MTHFR A1298C/MTHFR C677T; 4 of them showed simultaneous mutations of prothrombin G20210A/MTHFR C677T. Two patients in the latter group were homozygous for the MTHFR mutation. Two other patients had MTHFR A1298C and FVL G506A mutations simultaneously; 1 was homozygous for both mutations. Three patients had 3 simultaneous mutations. Two of them showed MTHFR A1298C/MTHFR C677T/FVL G506A mutations; 1 showed MTHFR A1298C/MTHFR C677T/ prothrombin G22010A mutations. The mean age of these 3 patients, all of whom were male, was 38.7 (5.13) years.

Thrombophilic Mutations and Platelet Count

In this research, we evaluated the platelet count from inpatients with thrombophilic mutations to observe a relationship between mutation type and platelet count, hoping to find an initial and simple approach to detect thrombophilic diseases. However, we observed no statistically significant difference in platelet count between patients with thrombophilic mutations and patients without mutations (**Table 3**). Also, no statistically significant difference was observed between the platelet count of patients with different numbers of simultaneous mutations and the no-mutation group.

Table 3. Platelet Count in Thrombotic Mutation Types[a]

Mutation	PLT (Mean [SD])
MTHFR A1298C	245.0 (84.80)
Prothrombin G20210A	269.0 (91.05)
Factor V Leiden G506A	195.5 (101.20)
MTHFR C677T	228.8 (70.65)
No mutation	253.3 (101.50)

Abbreviations: PLT, platelet; MTHFR, methylenetetrahydrofolate reductase.
[a]No statistically significant difference in platelet count was observed between patients with and without thrombophilic mutations. MTHFR *gene: OMIM 607093;* prothrombin *gene: OMIM 176930 for coagulation factor II or OMIM 188050 for thrombophilia due to thrombin defect.*
Accession number in GenBank: MTHFR(AY338232),FV (NM_000130), PT (M17262).

Discussion

In the present study, we have evaluated the prevalence of thrombophilic mutations in patients of Iranian ethnicity who have thrombophilia. Thrombophilic mutations were observed in 105 of 208 patients, including 13 with FVL, 51 with the MTHFR C677T mutation, 33 with the MTHFR A1298C mutation, and 8 with the prothrombin G20210A mutation. Eighteen patients showed 2 simultaneous mutations and 3 patients showed 3 simultaneous mutations. Bauduer and Lacombe[7] showed that the spectrum and variation of the most predominant prothrombotic mutations are underrepresented in sub-Saharan Africa, Indonesia, and Europe. FVL is the most frequent mutation; it is present in 50% of patients with familial thrombosis in the European population[13] However, FVL is absent in populations of individuals of sub-Saharan African and East Asian ethnicities, as well as the indigenous populations of the United Statesand Australasia.[7] The prothrombin G20210A mutation, the second-most-frequent thrombophilic mutation, is prevalent in whites, with a north-to-south gradient in Europe (prevalence of 0.017 and 0.030, respectively).[14] The third predominant thrombophilic mutation is the MTHFR mutation. The frequency of this mutation is higher than FVL or prothrombin 20210A mutations.[7] Of interest, in the present study, we observed the MTHFR

mutation, especially the C677T mutation, as occurring most frequently, followed by FVL and prothrombin 20210A (**Table 2**). These findings are in agreement with those of other researchers,[4,10,15] which show that FVL is the most common mutation in other populations. This may occur due to the biochemical role of vitamins B_{12}, B_6, and folic acid in MTHFR activities.

Also, our results showed a positive correlation of the A1298C mutation to the C677T and FVL mutations but a negative correlation with prothrombin G20210A. C677T gene mutation has a positive correlation with prothrombin G20210A and a negative correlation with FVL. The prothrombin G20210A gene mutation also has a negative correlation with FVL. Considering the results of our genetic and statistical analysis, we conclude that the 51 cases of MTHFR C677T and 33 of MTHFR A1298C mutations we observed in 208 patients with thrombotic disorders are the most common among our cohort, followed by FVL and prothrombin G20210A mutations. Similarly, the female patients in our cohort are younger, on the whole, than their counterparts in the cohorts of similar studies we reviewed. According to our results, platelet counts were in the normal range in patients with thrombophilia; no statistically significant difference was observed between platelet count and mutation type (**Table 3**).

Regarding the high prevalence of MTHFR C677T mutation in our region, family members of any patient with the MTHFR C677T mutation should be screened to identify this genetic mutation. Further investigations are needed to determine an exact reason for the regional variations of genetic thrombophilic factors and its importance in thrombogenesis.

The prevalence of FVL, prothrombin G20210A, MTHFR A1298C, and C677T mutations were scrutinized among patients of Iranian ethnicity who have thrombophilia. Prevalence of different thrombophilic mutations in ethnic Iranian patients can be related to nutritional regimen and genetic variation within the Iranian ethnicity. Additional genetic screening studies in different ethnic populations are required. LM

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