

Prevalence of Three Prothrombotic Genes Polymorphisms: Factor II, Factor V and MTHFR in Referred Population of Gujarat

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To study the prevalence of major mutations associated with Venous thromboembolism (VTE) in referred individuals at Gujarat.

Place and Duration of Study: PanGenomics International, Sterling Accuris Diagnostics, Ahmedabad, Gujarat, India between June 2020 to December 2021.

Methodology: 121 individuals referred for thrombosis genetic test were included in the study. Among 121 subjects, 71 were male and 50 were female. SNP genotyping was performed with melt curve analysis and confirmed with sanger sequencing.

Results: Highest prevalent SNP was of MTHFR A1298C with allelic frequency of 36.78%. Factor V and MTHFR SNP allelic frequency was comparable to previous studies. Absence of Factor II mutant allele in the studied population was inconsistent with the population from other countries but supports previous studies with Indian population. Minor allele frequency for MTHFR A1298C mutation was high as 36.78% and C677T mutation was 13.22%. Factor V Leiden R506Q allele frequency was 2.89% and H1299R allele frequency was 9.50%.

Conclusion: Among various surveys conducted throughout the world, considerable variation in

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prevalence of Factor II, Factor V and MTHFR gene mutations have been found. The mutations studied here are major risk factors for thrombosis. Population-based prevalence study of these mutations may help in reflecting the burden of disease in studied population.

Keywords: Factor II; Factor V; MTHFR; allelic frequency; melt curve; sanger sequencing.

1. INTRODUCTION

Venous thromboembolism (VTE) is a complex vascular disease with multifactorial pathogenesis that results in two major clinical manifestations, deep-vein thrombosis (DVT) and Pulmonary Embolism (PE) [1,2]. It is an inherited blood-clotting disorder, that increases the affected individual's tendency to form abnormal blood clots that may block blood vessels. VTE is the third leading cause of cardiovascular death across the globe [3]. As per Centers for Disease Control and Prevention (CDC) VTE is a leading cause of preventable hospital death in the United States. As many as 70% of cases of VTE are preventable. In the US, VTE affects an estimated 900,000 people each year, leading to an estimated 60,000–100,000 deaths (CDC). The identification of various factors that contribute for thrombosis, particularly the role of coagulation disorders, has expanded the knowledge of venous thrombosis over the past years [4]. As these abnormalities are found to be frequent in general population, they will be detected in large number of population at the same time. As per the studies carried out by Centers of Disease Control and Prevention, several factors such as surgery, pregnancy, oral contraceptives, cancer, antiphospholipid antibodies as well as hereditary factors interact to promote the development of deep vein thrombosis and pulmonary embolism.

Acquired as well as hereditary factors interact to promote deep vein thrombosis (DVT) and pulmonary embolism (PE). Acquired factors mainly include pregnancy, contraceptives, surgery, cancer, immobilization, antiphospholipid antibodies. Hereditary factors that induce VTE development can be classified as Protein C and Protein S deficiencies and polymorphisms [5]. In recent years, Factor V, MTHFR, and Prothrombin mutations were found to be responsible for producing Activated Protein C Resistance (APC R), hyperhomocysteinemia and increasing levels of Factor II respectively [6].

Factor V Leiden is a genetic disorder where activity of activated Protein C against coagulation factor V diminish which increases the risk for VTE. The F5 gene provides instructions for

making a protein called coagulation factor V; this protein plays a critical role in the coagulation cascade. Variant detected in the F5 gene is a single nucleotide polymorphism (SNP) located at exon 10. A missense substitution of base G to base A changes the protein's amino acid from arginine to glutamine (c.G1691A or p.R506Q). Since this amino acid is normally the cleavage site for APC, the mutation prevents efficient inactivation of factor V. The coagulation system is controlled by several proteins, including one called activated protein C (APC). Under normal circumstances, APC inactivates coagulation factor V and which slows down the clotting process, preventing clots from growing too large. However, in people with factor V Leiden thrombophilia, coagulation factor V cannot be inactivated normally by APC. As a result, the clotting process remains active longer than usual, increasing the chance of developing abnormal blood clots [7].

MTHFR (methylenetetrahydrofolate reductase) is an enzyme involved in the metabolism of folate and homocysteine [8]. It plays a role in maintaining cellular folate levels. It is also a cofactor needed to convert homocysteine to methionine. Two common variants have been characterized that reduce the function of the MTHFR enzyme [9]. These are the c.C677T variant which is a change from cytosine to thymine at position 655 within the gene and the c.A1298C variant which is a change from adenine to cytosine at position 1286 within the gene. Reduced MTHFR enzyme activity can lead to elevated homocysteine levels, which is a known independent risk factor for development of cardiovascular disease and venous thrombosis. Reduced enzyme function can also affect folate levels and risk for recurrent pregnancy loss and neural tube defects in the fetus [10].

Coagulation factor II is the precursor of thrombin, which participates as a serine protease (factor II) in the coagulation cascade. The gene that codes for prothrombin has been mapped on chromosome 11 at position 11p11-q12. The prothrombin c.G20210A mutation is associated with a 3-fold increase in the risk of VTE. High Factor II plasma levels associated with this

mutation are believed to be responsible for the increased risk of VTE [11]. According to the findings, Factor II plasma levels have a considerable influence on thrombin production, which is important for hemostasis and thrombosis.

The prevalence of these SNPs varies depending on geographical location and ethnic background of the population. Contrary to earlier belief, the incidence of VTE in India is comparable to that in Western countries. In the present study, allelic frequency of polymorphisms related to VTE is investigated in referred individuals from Gujarat. To the best of our knowledge, there are no previous reports which assess these five SNP frequencies dedicatedly in the Gujarat population.

2. MATERIALS AND METHODS

2.1 Sample Collection

Blood samples were collected from 121 referred individuals at Sterling Accuris Diagnostics laboratory from all the different regions of Gujarat. Patients Aged >18yrs and referred by a physician for the test, were included in the study. Approximately 3-4ml blood was obtained from the individuals in a labeled sterile ethylenediaminetetraacetate (EDTA) vacutainer and were kept at -4°C for DNA extraction.

2.2 DNA Extraction

Genomic DNA isolation was done from the blood samples using QIAamp DNA Mini Kit. DNA was extracted from 200 µl using whole blood using the spin columns provided in the kit. DNA was quantified using Qubit 2.0 fluorimeter with Qubit dsDNA HS assay kit as per instructions provided in the kit manual. DNA working solution of 100ng/µl was prepared for each sample and used further for PCR and sequencing. The isolated DNA stock and working solution were stored at -20°C.

2.3 Sanger Sequencing Based Detection of Mutations

The primers listed in Table 1 were used for PCR amplification Factor II, Factor V and MTHFR genes products. All the primers were tagged with M13 sequence at 5' end. PCR products were treated with Exosap-IT at 37°C for 15min and 80°C for 15 min using ABI Veriti thermal cycler. Cycle sequencing was performed with M13 primers using the following reaction mix - 1.8µl of 5X sequencing buffer, 0.5 µl of RR100 (BDT), 1 µl of M13 forward or reverse primer, 0.5 µl of Exosap treated product and 6.2 µl of milliQ. Cycle sequencing products were analyzed with ABI3500 Genetic Analyzer. Further, the sequences obtained were then analyzed using Chromaslite software, by comparing them with the reference sequences.

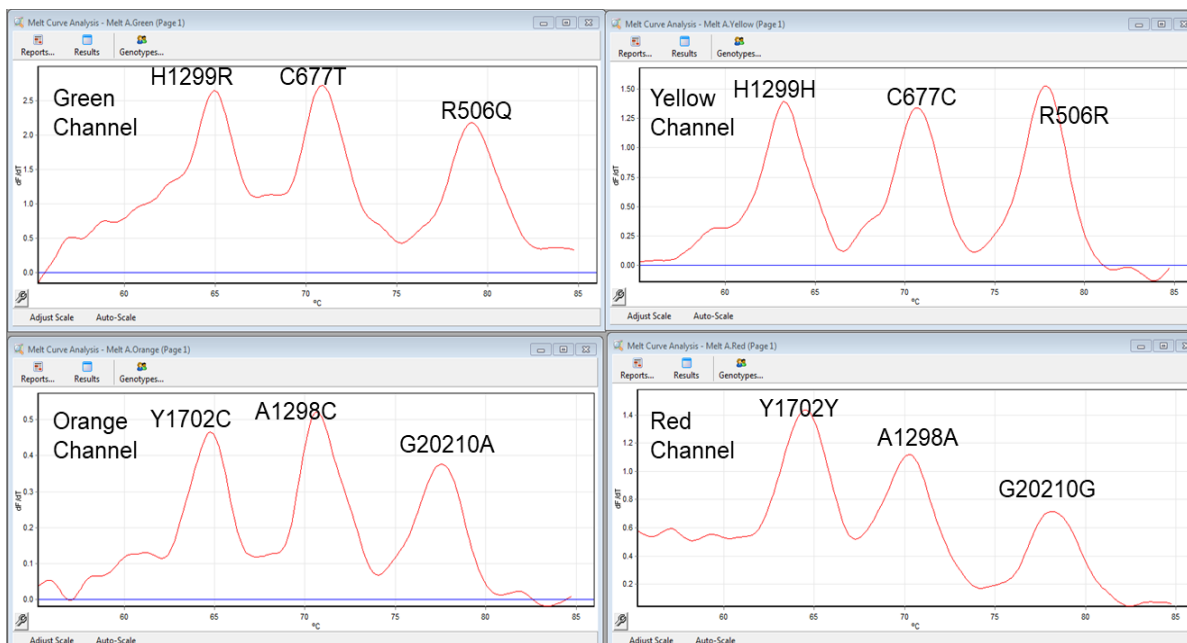


Fig. 1. Melt-curve analysis of Anyplex™ II thrombosis SNP panel assay

Table 1. Primers used in the study

Gene	Primer Name	Primer Sequence (5'-3')	Product Size
Factor II	FTR-II-1F	5'TGAAAACGACGGCCAGTATGTGTTCCGCCTGAAGAAG3'	275 bp
	FTR-II-1R	5'CAGGAAACAGCTATGACCAGTGCTCGGACTCCAGCGT3'	
Factor V	FTR-V-1F	5'TGAAAACGACGGCCAGTTGTTATCACACTGGTGCTAA3'	345 bp
	FTR-V-1R	5'CAGGAAACAGCTATGACCGATCAGAGCAGTTCAACCA3'	
MTHFR	MTHFR-1F	5'TGAAAACGACGGCCAGTCTCAAGGCAGGACAGTGT3'	581 bp
	MTHFR-1R	5'CAGGAAACAGCTATGACCAAGAAGCTCAGCGAACTCAGCAC3'	
	MTHFR-2F	5'TGAAAACGACGGCCAGTGCAGACCTTCCTTGCAAATAC3'	491 bp
	MTHFR-2R	5'CAGGAAACAGCTATGACCTTGCCATGTCCACAGCAT3'	
M13	M13 – F	5'TGAAAACGACGGCCAGT3'	
	M13 – R	5'CAGGAAACAGCTATGACC3'	

2.4 Real-Time PCR Based Detection of Mutations

Anyplex™ II SNP panel assay is a real-time PCR based assay. This assay was used for the study of the six thrombosis-associated single nucleotide polymorphisms (SNPs): Factor II (G20210A), Factor V (R506Q, H1299R, Y1702C) and MTHFR (C677T, A1298C). Real-time PCR detection of these SNPs was performed using Qiagen real-time thermal cycler- Rotor-Gene Q. A total system of 20 µl per reaction was set which incorporated 15 µl of master mix and 5 µl of DNA template. The thermal cycle and fluorophores were set according to the protocol provided by the kit manufacturer. Once the amplification cycles were completed, the mutations were determined by melt curve analysis as represented in Fig. 1.

2.5 Statistical Analysis

Differences in mutation prevalence between gender were tested for heterogeneity by the chi-square test using PSpP ver3 software. A p-value of less than 0.05 was considered as statistically significant. The prevalence of mutations was calculated as percent (%). Age is expressed as the mean±SD.

3. RESULTS

Among 121 subjects, 71 were male and 50 were female with ranging in age from 18 to 86 with a mean age of 38.6 ± 14.6 . Among three genes, MTHFR allele mutation was the most prevalent. From a total of 121 samples, 53 samples were heterozygous and 13 samples were homozygous for A1298C mutation (Table-2). Thus, mutant allele for A1298C was widely present in Gujarat population accounting for allelic frequency of 36.78% (Table-3). Fig. 2 depicts representative electrogram of wild type and mutant alleles found in the study. MTHFR C677T mutation was also highly prevalent with 16 heterozygous and 3 homozygous cases. The allele frequency was 13.22% for C677T mutation. Also, 8 out of 121 cases were heterozygous and 1 case was homozygous for both the mutation. Upon analyzing the data from Table-2 with PSpP software, there was no difference among male and female allelic frequency. Pearson Chi-square test p-value for MTHFR mutation Vs. gender was 0.065.

Surprisingly, none of the samples were found positive for Factor II mutation. 21 individuals were found with H1299R heterozygous mutation and only 1 individual was homozygous. In case of R506Q mutation, 7 cases were heterozygous and there was no homozygous case. Thus Factor V mutant allele was less prevalent compared to MTHFR mutation. Allelic frequency of H1299R was 9.50% and R506Q was 2.89%. There was no significant difference in mutation between male and female (p-value 0.409, chi-square test)

The minor allele frequency was also calculated in this study. From the data presented in Table-3, the frequency of 13.22% for C677T and 36.78% for A1298C was obtained. Table-3 also represents mutant allele frequencies obtained in the ALFA project and 1000Genomes project for south Asian population. Factor V mutant allele frequency obtained in the current study was 2-4 fold higher than the database frequency. For Factor II and MTHFR, mutant allele frequency is comparable to that of database frequency.

Haplotype frequency for MTHFR gene and Factor V gene mutations were studied as shown in Table-4. Major Haplotypes i.e TGTC, GGTC, TATC, GGCC frequencies were comparable to frequency data obtained from LDlink tool (NCI) which used data from 1000Genomes project. Haplotype GATC, which represented double heterozygous mutation for MTHFR SNPs, was observed at higher rates compared to data obtained from LDlink for south Asian population. Moreover, Linkage disequilibrium data for South Asian population was analyzed using LDmatrix tool (NCI) (Fig. 3). SNPs of the same gene showed high correlation for linkage disequilibrium ($D' = \sim 1$) as these SNPs were only few base pairs apart on chromosome no.1. MTHFR SNPs Vs. Factor V SNPs were approximately 1.5 Mega base pairs (Mb) apart on chromosome no.1. D' value for MTHFR SNP Vs. Factor V SNP was in the range of 0.1-0.2 as indicated in Fig. 3 which represented linkage equilibrium state.

4. DISCUSSION

Several studies have been conducted demonstrating considerable variations of the prevalence of venous thromboembolism associated genes. Major genetic cause for VTE accounts for polymorphisms with Factor II (G20210A), Factor V (R506Q, H1299R) and MTHFR (C677T, A1298C) genes [6].

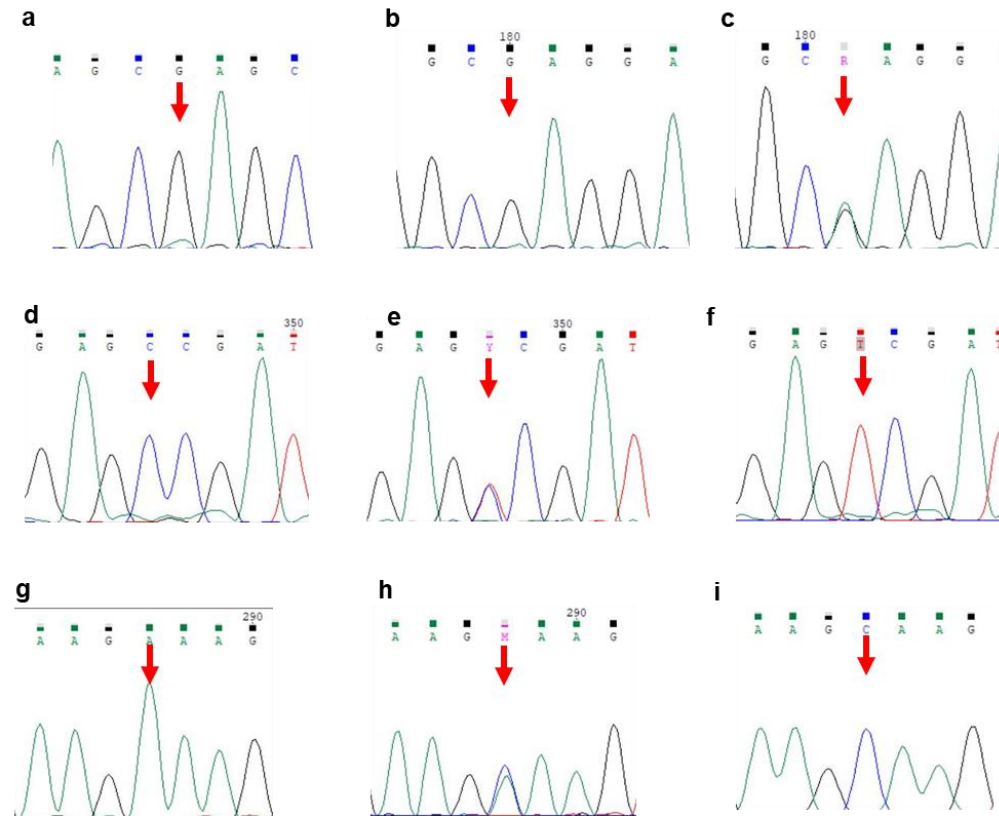


Fig. 2. Image electrogram of Factor II, Factor V and MTHFR genes point mutations

(a) Factor II WT, (b) Factor V WT, (c) Factor V R506Q heterozygous, (d) MTHFR C677T WT, (e) C677T heterozygous, (f) C677T homozygous, (g) MTHFR A1286C WT, (h) A1298C heterozygous, (i) A1298C homozygous

Table 2. Distribution of thrombotic genes mutation in Gujarat Population

MTHFR	Female	Male	Total
WT	7	20	27
A1298C(HET)	25	28	53
A1298C(HM)	5	8	13
C677T(HET)	10	6	16
C677T(HM)	0	3	3
Compound mutation			
C677T(HET), A1298C(HET)	3	5	8
C677T(HM), A1298C(HM)	0	1	1
Total	50	71	121
Factor V			
WT	37	55	92
H1299R(HET)	10	11	21
H1299R(HM)	0	1	1
R506Q(HET)	3	4	7
Total	50	71	121
Factor II			
WT	50	71	121

Table 3. Allelic frequency of Factor II, Factor V and MTHR genes SNPs

SNP	Minor allele frequency	Allelic frequency ALFA (NCBI dbSNP, dbGaP)	Allelic frequency 1000Genomes
MTHFR	A1298C	C (0.37)	0.417
	C677T	T (0.13)	0.119
Factor II	G20210A	A (0.00)	0.00
Factor V	R506Q/G1691A	A (0.029)	NA
	H1299R	A (0.095)	0.064

Table 4. Haplotype frequency of MTHFR and FactorV SNPs

rs1801131	rs1801133	rs1800595	rs6025				
MTHFR	MTHFR	Factor V	Factor V	Haplotype	Haplotype Count (Total 121 X 2)	Observed Haplotype frequency	Reported Haplotype LDlink Tool (NCI)
A1298C/ T1298G (T Wild Type Allele)	C677T/ G677A (G Wild Type Allele)	H1299R (T Wild Type Allele)	R506Q.(C Wild Type Allele)				
T	G	T	C	TGTC	123	0.5083	0.4346
G	G	T	C	GGTC	64	0.2645	0.3824
T	A	T	C	TATC	18	0.0744	0.1094
G	G	C	C	GGCC	12	0.0496	0.0297
G	A	T	C	GATC	7	0.0289	0.0010
T	G	T	C	TGCC	5	0.0207	0.0256
G	A	C	C	GACC	3	0.0124	-
G	G	T	T	GGTT	3	0.0124	-
T	A	C	C	TACC	3	0.0124	0.0061
T	G	T	T	TGTT	3	0.0124	0.0051
T	A	T	T	TATT	1	0.0041	0.0020

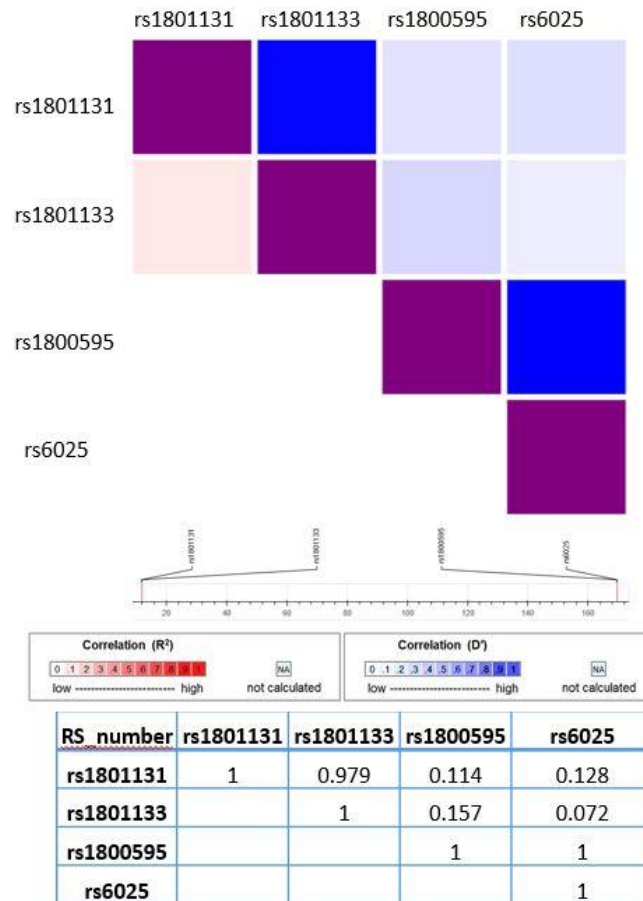


Fig. 3. Linkage disequilibrium matrix for SNPs of Factor V and MTHFR genes using LDmatrix tool

Values in table indicates linkage correlation values (D') for the respective SNPs

Factor V Leiden, rs6025 (c.G1691A or p.R506Q), is point mutation and most frequently associated with VTE. Heterozygous individuals have 5 times and homozygous individuals have 10 times higher risks of developing VTE. European population have mutant allele frequency of 4-7% with highest prevalence in Greece (7%) [12]. West Bank population has high mutation frequency of 11% [13]. In a study conducted on South Indian population, 8 out of 240 samples (3.33%) found positive for Factor V Leiden [14]. Factor V gene polymorphism (His1299Arg; also named HR2) has also been reported as a risk factor for the development of VTE. H1229R mutant allele frequency in Lebanese population was around 6% [15] and Saudi-Arabia population had 5% [16]. There are no reports to our knowledge, indicating H1229R mutation prevalence in Indian population. In our study, Factor V Leiden, R506Q, mutant allele frequency was 2.89% and H1299R allele frequency was 9.50%.

Factor II polymorphism (G20210A) is associated with a higher risk of venous and arterial thrombosis in various studies. The polymorphism causes high plasma levels factor II which is a coagulation factor. There is 2.8 times higher risk of VTE in individuals with factor II polymorphism. In a study conducted in Argentina, 2.6% of samples were heterozygous for G20210A mutation [17]. In Indian Tamilian population, none of the samples had mutant allele among 72 subjects tested. In our study as well, mutant allele for G20210A mutation was not detected. The ethnic background of Indians, as well as geographical locations, are plausible factors resulting in the complete absence of mutant allele for Factor II in both the studies.

Mutation c.C677T and c.A1298C in MTHFR gene results in a thermolabile variant with reduced enzymatic activity. Decreased MTHFR enzyme activity slows down the homocysteine-to-methionine conversion process and can lead to a

buildup of homocysteine in the blood. MTHFR mutation is found to increase VTE risk by 2.5-fold [10,18]. A study conducted by All India Institute of Medical Sciences found mutant allele frequency of MTHFR C677T and A1298C was 15% and 44% respectively in Indian population [19]. A targeted study for C677T mutation at Eastern Uttar Pradesh found T allele frequency of 12% [20].

5. CONCLUSION

The SNPs analyzed in the present study, are major genetic risk factors for vascular diseases leading to thrombosis. Thrombosis may lead to several complications such as pulmonary embolism, cardiovascular disorders and pregnancy-related conditions such as fetal growth retardation and even stillbirth. The minor allele frequency for Factor V R506Q mutation was 2.9% and H1299R was 9.5%, for MTHFR C677T mutation was 13.22% and A1298C was 36.78%. Mutant allele for Factor II G20210A was not observed. The present study in referred subjects of Gujarat may contribute to a better understanding of genetic risk factors and burden of the thrombosis and other related disorders.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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