Factor V Leiden, Prothrombin 20210G>A, MTHFR 677C>T and 1298A>C, and Homocysteinemia in Tunisian Blood Donors

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Specific genetic conditions are known to be associated with high risk of venous thromboembolism. This genetic basis varies widely between ethnic groups. We investigated the distribution of four inherited polymorphisms in 113 unselected Tunisian blood donors by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The allele frequencies of Factor V Leiden (FVL), prothrombin 20210G>A, methylenetetrahydrofolate reductase (MTHFR) 677C>T, and MTHFR 1298A>C mutations were 3, 0.9, 30, and 31%, respectively. The MTHFR

677C>T polymorphism was influenced by age. Twenty-nine of the 113 blood donors demonstrated more than one genetic markers. Hyperhomocysteinemia was found in 12 subjects, and it was statistically associated to the MTHFR 677TT genotype. Principal component analysis allowed disclosing the resemblance between Mediterranean populations. Our findings may be helpful for population genetics study, and provide epidemiologic database for further studies in thrombosis field among Tunisians. *J. Clin. Lab. Anal.* 26:167–173, 2012. © 2012 Wiley Periodicals, Inc.

Key words: Factor V gene; prothrombin gene; methylenetetrahydrofolate reductase; homocysteine; Tunisia

INTRODUCTION

Venous thromboembolism (VTE), including deep venous thrombosis and pulmonary embolism, remains a major worldwide public health problem, which affects annually approximately 1–3 per 1,000 persons in the general population (1, 2). It is an important cause of mortality and morbidity, especially in the elderly (1, 3, 4). VTE is a multifactorial disease due to the interplay of a variety of acquired and genetic traits in combination with environmental factors such as aging, obesity, prolonged immobilization, surgery, trauma, oral contraceptives, and cancer (1, 3, 5).

The genetic predisposition to thrombosis is influenced by the ethnicity of the population, which varies from country to country and is closely related to social and political characteristics of the settlement (6, 7). Single mutations in coagulation Factor V [1691G>A or Factor V Leiden (FVL)] and prothrombin (20210G>A) genes emerged as one of the major inherited risk factors for venous thrombosis. The Factor V gene mutation, predicting replace-

ment of Arg506 with Gln, confers resistance to the anticoagulant action of activated protein C (APC), which leads to a hypercoagulable state with ten- and 100-fold increased risk of venous thrombosis in heterozygous and homozygous carriers, respectively (8, 9). The GNA transition at nucleotide position 20,210 in the prothrombin gene is associated with a slight increase in plasma levels of prothrombin, which results in a hypercoagulable state and a lifelong three- to fourfold increased risk of venous thrombosis (2, 10). In addition, two genetic variants, 677C>T and 1298A>C, of the methylenetetrahydrofolate reductase (MTHFR) have been reported to reduce the enzyme activity, possibly resulting in increased levels of

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homocysteine. While these two MTHFR polymorphisms are not consistently associated with thrombosis, elevated homocysteine is thought to be a risk factor for venous thrombosis (11, 12). However, hyperhomocysteinemia is multifactorial and disruptions in homocysteine metabolism may be due to other genetic factors or nutritional deficiencies (11, 13). A possible cumulative effect on VTE risk by the simultaneous transmission of the major genetic traits mentioned above has been supported by some studies (14, 15) but not by other recent reports (16, 17). Previous immunogenetic studies showed that Tunisian population was composed of a mixture of different ethnicities as a result of active movement of population (18, 19). However, there are no reports to date associating the four genetic prothrombotic traits, nor their thrombosis relevance.

The aim of this study was to evaluate the prevalence of the above-mentioned polymorphisms in the Tunisian population in order to provide baseline epidemiological data for future clinical investigations in the field of VTE and other clinical entities.

MATERIALS AND METHODS

Subjects and Blood Sample Collection

Blood samples were collected in K3 ethylenediaminetetraacetic (EDTA) tubes from 113 unselected and unrelated healthy Tunisian blood donors [55 men (49%); 58 women (51%); mean age: 31.2 ± 8.1 years; range: 19–56] originating from the center of Tunisia with their written informed consent. No familial history of thromboembolism was noted among blood donors. The blood samples were promptly centrifuged (10 min at 3,000 rpm at $+4^{\circ}\text{C}$) and the plasma was separated and stored at -80°C for subsequent homocysteine measurement within 2–4 weeks, while the buffy coat was used for polymorphism studies. Genomic DNA was extracted from 300 μl of buffy coat using a commercially available DNA isolation kit (Wizard[®] Genomic, Promega, Madison, WI).

Detection of FVL Mutation

Extracted genomic DNA was tested for the presence of FVL mutation using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). In brief, a 247 base pairs (bp) segment of the Factor V gene was amplified using specific primers (5'-TTT CTT TCA GGC AGG AAC AAC ACC AGA ATC-3' and 5'-GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT-3'). The PCR product (10 μl) was digested with restriction enzyme *Hind* III at 37°C for 16 h. The digested amplicon from wild-type DNA gives one band of 247 bp,

heterozygous FVL mutation shows 247 and 214 bands, while homozygous FVL gives the single 214-bp band.

Detection of Prothrombin 20210G>A Mutation

The prothrombin 20210G>A mutation was tested by PCR-RFLP method. In brief, a 345-bp genomic DNA fragment encompassing a part of the prothrombin gene that contains the mutation was amplified by PCR using specific primers (5'-TCT AGA AAC AGT TGC CTG GC-3' and 5'-ATA GCA CTG GGA GCA TTG AAG C-3'). The PCR product (5 μl) was digested with *Hind* III, at 37°C for 16 h. In this method, the wild-type DNA yields a solitary 345-bp band, heterozygous 20210G>A mutation yields two bands of 345 and 322 bp, and homozygous mutation only one band of 322 bp.

Digested DNA fragments for both FVL and prothrombin 20210G>A were then separated on the same 6% polyacrylamide gel.

Detection of MTHFR (677C>T and 1298A>C) Mutations

MTHFR genotype analysis was performed by PCR-RFLP analysis using *Hinf* I and *Mbo* II digestion for 677C>T and 1298A>C detection, respectively. The primer sequences for 677C>T were: forward, 5'-TGA AGG AGA AGG TGT CTG CGG GA-3', and reverse, 5'-AGG ACG GTG CGG TGA GAG TG-3'. The 677C>T mutation introduces a new *Hinf* I restriction site that results in the digestion of the 198-bp amplicon into 175- and 23-bp fragments. The MTHFR 1298A>C polymorphism was analyzed in similar conditions as 677C>T mutation using the following primer set: forward, 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3', and reverse, 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'. By abolishing a *Mbo* II restriction site, the homozygous 1298A>C mutation results in the digestion of the 163-bp amplicon into 84-, 31-, 30-, and 18-bp fragments, while the wild-type variant is digested into 56-, 31-, 30-, 28-, and 18-bp fragments. Digested DNA fragments were then separated on 6% and 15% polyacrylamide gel for 677C>T and 1298A>C, respectively.

Plasma Homocysteine Concentration

The nonfasting plasma concentration of total homocysteine (tHcy) was measured by using a fluorescence polarization immunoassay method according to the manufacturer's instructions (Abbott AxSYM Homocystein, Axis-Shield Diagnostics, UK). This method is in good correlation with the high-performance liquid chromatography assay.

TABLE 1. Genotype and Allele Frequencies of Factor V Leiden, Prothrombin 20210G>A, MTHFR 677C>T, and MTHFR 1298A>C Mutations in 113 Tunisian Blood Donors

	Genotype frequency			Mutation prevalence (%)	Allele frequency (%)
	Wild, <i>n</i> (%)	Heterozygote, <i>n</i> (%)	Homozygote, <i>n</i> (%)		
Factor V Leiden	107 (94.7)	5 (4.4)	1 (0.9)	5.3	3
Sex (M/F)	53/54	2/3	0/1		
Prothrombin 20210G>A	111 (98.2)	2 (1.8)	0	1.8	0.9
Sex (M/F)	54/57	1/1			
MTHFR 677C>T	58 (51.3)	42 (37.2)	13 (11.5)	48.7	30
Sex (M/F)	26/32	22/20	7/6		
MTHFR 1298A>C	59 (52.2)	38 (33.6)	16 (14.2)	47.8	31
Sex (M/F)	34/25*	18/20	3/13**		

*Significant difference ($P = 0.047$); **Significant difference ($P = 0.01$). M, male; F, female.

Statistical Analysis

Allele and genotype frequencies were calculated by direct counting method and their consistency with Hardy–Weinberg equilibrium was evaluated by a chi-square goodness-of-fit test.

For comparison of means and medians of tHcy levels, Student's *t*-test and Mann–Withney nonparametric test were used, respectively. Results were expressed as mean $\pm$ SD or median $\pm$ SD.

Chi-square test for contingency tables was used to compare paired frequencies. Statistical analysis was performed using SPSS (V11.0) software. $P < 0.05$ was considered to be statistically significant.

In order to assess the relatedness between the allele frequencies of the four inherited mutations in different populations, a principal component multivariate analysis (PCA) (20) was performed using the statistical STASTICA 4.3 software package.

RESULTS

Allele Frequency of the Four Inherited Thrombophilic Traits

The prevalence and allele frequencies of FVL, prothrombin 20210G>A, and both MTHFR mutations are summarized in Table 1. The distribution of the studied genotypes was in Hardy–Weinberg equilibrium. The PCA showed a global view for the relatedness between 20 different populations according to the allele frequencies of the four inherited mutations (Fig. 1).

MTHFR Polymorphism and Homocysteinemia

We classified the subjects into two age groups: younger (<40 years; $n = 85$) and older (≥ 40 years; $n = 28$). As shown in Table 2, the MTHFR 677C>T mutation frequency decreased as the ages of the subjects increased.

Similar effect was found with the 1298A>C mutation, but the difference was not significant. No double homozygous 677TT/1298CC was detected.

The mean tHcy level was significantly higher ($P = 0.03$) in males as compared to females (mean $\pm$ SD: 13.04 ± 5.2 vs. 11.14 ± 4). The effect of combined MTHFR genotypes on the tHcy level is shown in Figure 2. Significantly higher tHcy median values were associated with the 677TT genotype. There was a synergistic effect of the 1298AC genotype on the tHcy level in homozygotes 677TT [677TT/1298AC: 19.53 ± 9.9 (Median $\pm$ SD) vs. 677TT/1298AA: 15.14 ± 6.6], even though it was not statistically significant ($P = 0.64$).

Combined Mutations

Eighty-eight of the 113 blood donors showed at least one inherited mutation (77.9%). Among them, 29 (25.7%) have combined mutations: one was triple heterozygous FVL/MTHFR 677CT/MTHFR 1298AC; one was double homozygous FVL/MTHFR 1298CC; one was heterozygous FVL and homozygous MTHFR 677TT; one was heterozygous FVL and homozygous MTHFR 1298CC; one was double heterozygous FVL/1298AC; one was triple heterozygous prothrombin 20210GA/MTHFR 677CT/1298AC; one was double heterozygous prothrombin 20210GA/MTHFR 677CT; 16 were double heterozygous MTHFR 677CT and 1298AC; four were homozygous MTHFR 677TT and heterozygous MTHFR 1298AC; and two subjects were heterozygous MTHFR 677CT and homozygous MTHFR 1298CC.

DISCUSSION

Our findings show that the allele frequency of FVL, prothrombin 20210G>A, MTHFR 677C>T, and MTHFR 1298A>C mutations in Tunisian blood donors were 3,

Fig. 1. Principal component multivariate analysis (PCA) showing a global view of the relationship among 20 different populations according to the allele frequencies of the four inherited thrombophilia mutations (FV Leiden, prothrombin 20210G>A, MTHFR 677C>T, and MTHFR 1298A>C). PCA associates both geometrical and statistical analysis of the allele's variance. Its bidimensional representation is performed according to the axis 1 and 2 that are the two first dimensions of the correspondence analysis that explain 87.65% of the total inertia. Populations data are taken from "The allele Frequency Database: <http://alfred.med.yale.edu/>" and references (5, 21–28) and (40–49).

TABLE 2. Distribution of MTHFR 677C>T and 1298A>C Mutations According to Age

	Age		<i>P</i>
	<40 years (<i>n</i> = 85)	≥40 years (<i>n</i> = 28)	
MTHFR 677C>T			
Positive	47 (55.3%)	8 (28.6%)	0.014
CT	36 (42.4%)	6 (21.4%)	0.047
TT	11 (12.9%)	2 (7.1%)	0.40
MTHFR 1298A>C			
Positive	40 (47.1%)	14 (50%)	0.78
AC	30 (35.3%)	8 (28.6%)	0.51
CC	10 (11.8%)	6 (21.4%)	0.20

0.9, 30, and 31%, respectively. Coexistence of multiple polymorphisms was demonstrated in 29 of them.

The prevalence of inherited thrombophilia mutations has been determined in different groups and races, observing a variation that considers whether the population in study is Caucasian, Asian, African, or an ethnic minority. According to the PCA construction, the four inherited abnormalities could be clustered into two major areas. The first area encompasses Caucasians and Mediterraneans of the eastern seashore, where the four mutations are prevalent. In Europe, the allele frequencies of FVL, prothrombin 20210G>A, MTHFR 677C>T,

and MTHFR 1298A>C vary between 0% in Greenland to 7% in Greece (21), 0.6% in England (23) to 4% in Spain (24), 24.5% in Germany to 43.8% in Italy (25), and 22% in Poland to 36% in Netherlands (26), respectively. Similarly, in Middle East, high frequency of FVL [Cyprus 7.2%, Lebanon 7.9%, Jordan 8.5%, Turkey 4.1% (22)], prothrombin 20210G>A [Cyprus 3.9% (22), Lebanon 1% (23, 24), Jordan 1% (22), Turkey 1.3% (22)], MTHFR 677C>T [Cyprus 40% (22), Lebanon 30.4% (22), Jordan 25.3% (27), Turkey 28.3% (27)], and MTHFR 1298A>C [Cyprus 41% (24), Lebanon 49% (24), Jordan 31.5% (27), Turkey 40.1% (27)] was demonstrated.

In opposite, these inherited abnormalities are rare among Asians and black Africans, who define the second area. In Asians, the FVL and the prothrombin 20210G>A mutations are quasi-nonexistent, and the prevalence of MTHFR polymorphism, which can exceed 50% in white populations, is less than 20% in Asians and Africans (25–27).

Epidemiological data concerning the genetic risk factors are scarce in African countries. In sub-Saharan region, the allele frequency of FVL and prothrombin 20210G>A is as low as 0% (5, 21) and those of the MTHFR 677C>T and 1298A>C polymorphisms are 6.6% (25) and 13% (28), respectively. In the Maghreb, corroborating data for FVL, prothrombin 20210G>A, and MTHFR 677C>T mutations were reported in Morocco

Fig. 2. Distribution of total homocysteine plasma concentrations according to the genotypes of methylenetetrahydrofolate reductase. Significant high median values were found in TT/AA (15.14 $\mu\text{mol/l}$) and TT/AC (19.53 $\mu\text{mol/l}$) compared to CC/AA genotypes (10.88 $\mu\text{mol/l}$). The median is indicated as solid lines.

[0, 1.2, and 28.9% (22), respectively] and in Algeria [1, 1.4, and 34.3% (29), respectively], but there is no available data for MTHFR 1298A>C polymorphism. These Maghrebian findings are quite similar to Caucasians data (5, 25–28).

Our study showed that the frequency of MTHFR 677C>T decreased as the ages of the subjects increased, which is in accordance with other studies (30, 31). This finding was not showed with the MTHFR 1298A>C polymorphism. We found no double homozygotes (677TT/1298CC). In contrast, we found four individuals with the 677TT/1298AC genotype and two others with the 677CT/1298CC genotype reinforcing the suggestion that the occurrence of these two polymorphisms in cis is not a rare feature (32, 33). We showed that hyperhomocysteinemia was closely correlated to the homozygous 677TT genotype and there was a synergistic effect by the co-inheritance of the 1298AC genotype. Our results are in accordance with previous reports (33, 34). It is well established that the both mutations in MTHFR gene result in two biochemically different enzymes, with less reduced enzyme activity with 1298A>C than 677C>T polymorphism (34). However, one potential limitation of our tHcy assessment was that we measured nonfasting homocysteinemia, and we did not consider environmental

influencing factors such as folate and vitamin-B12 status (11, 13).

The mechanism of the concomitant distribution of the four polymorphisms requires comment. It may be due to the chance or caused by a selecting process following independent founder mutations, which may arise among Caucasians and extend to other populations through migration and mixing of populations (5, 13).

On the other hand and as a consequence of the concomitant distribution of the four genetic markers, one might expect that Asians and Africans would experience less thrombosis events than predominantly white populations. Curiously, epidemiological data about thrombosis showed that this statement is not absolute. White et al. (35) demonstrated a quite heterogeneous and gradually decreasing annual incidence of idiopathic venous thrombosis according to the ethnicities; and it found to be 29, 23, 14, and 6 per 100,000 among African Americans, Caucasians, Hispanics, and Asians, respectively. In a second study, the authors confirmed that African Americans comprise the highest risk for thrombosis, while the risk for Hispanics is about half that of the Caucasians (36). In this respect, Klatsky et al. (37), by analyzing the data obtained from a large cohort of 128,934 persons, demonstrated that blacks are at higher risk, as compared

to whites, and that Asians are at lower risk. The low risk among Asians was further confirmed by several reports from Asian countries (38, 39).

So, according to these data, black populations living in western countries seem to be the most exposed to thrombosis risk despite the genetic protection conferred by the low prevalence of these four genetic markers. This discrepancy may reinforce, at least in part, the role of possible other hereditary polymorphisms not yet discovered and/or environmental influences including the lifestyle traits, prevalence of obesity, psychosocial stress, socioeconomic status, which may have remarkable impact on the risk of VTE within black populations. However, all these studies were undertaken in black Africans living in developed countries but the incidence of thrombosis remains yet unknown among native Africans. Further contributive epidemiological data in African countries including Maghrebians are required to highlight this issue.

CONCLUSION

To our knowledge, our study is the first Tunisian genetic report analyzing the simultaneous transmission of these four major polymorphisms. It demonstrates that the genetic profile of FVL, prothrombin 20210G>A, MTHFR 677C>T, and MTHFR 1298A>C is quite similar to Western population, Maghrebians, and Middle Eastern population. Combined inheritance of more than one of these four polymorphisms was found in nearly the quarter of blood donors, and would be with pejorative impact on VTE occurrence. These findings may be helpful for population genetics. Further investigations on the role of these mutations in the thrombosis pathogenesis among Tunisians are needed.

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