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Analysis of factor V Leiden and prothrombin mutations in patients with suspected thrombophilia in São Paulo state-Brazil

Análise da mutação no fator V de Leiden e na protrombina em pacientes do estado de São Paulo, Brasil

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ABSTRACT

Introduction: Prothrombin (factor II) is a thrombin precursor, which induces fibrin formation. A mutation in the prothrombin gene (G20210A) has been described, which is directly associated with high prothrombin levels, hence thrombophilia. G1691A mutation in the factor V Leiden (FVL) gene occurs on exon 10, one of the main cleavage sites for protein C activation, resulting in protein alteration. **Objective:** To identify and estimate the genotype frequency of the three possible genotypes and the frequency of the two existing alleles in the FVL and prothrombin genes in patients with suspected thrombophilia in the state of Sao Paulo. This study may provide more literature and reference data on the incidence of prothrombin genotypes among individuals in Brazil. **Material and methods:** Analysis of point mutation by real time polymerase chain reaction (RT-PCR). **Results:** We obtained a total of 100 individuals, from which 94% had the homozygous G genotype. Only 6% had heterozygous genotype and there was no individual with the homozygous genotype A for FVL gene. As to the prothrombin gene, the frequency was 97% for homozygous G genotype and 3% for the heterozygous genotype. There was no patient with the homozygous A genotype. **Conclusion:** This study demonstrated that the genotype identification of these genes is advisable for patients with suspected thrombophilia in this region.

Key words: factor V Leiden; G1691A mutation; prothrombin; G20210A mutation; real-time PCR; SNP.

INTRODUCTION

Thromboembolic diseases, due to their cosmopolitan character, are among the most frequent causes of morbidity, incapacitation and mortality, with a general incidence of one in 1,000 individuals annually[15, 20]. Venous and arterial thromboembolism is a hereditary or acquired disease that affects proteins from the anticoagulant system, thus leading to the formation of thrombi. Hereditary thrombophilias are generally caused by mutations in the genes that codify coagulation factors. G1691A mutation in factor V Leiden (FVL) and G20210A mutation in the prothrombin gene are the most prevalent causes of hereditary thrombosis[4, 14, 27].

FLV mutation consists in the exchange of guanine (G) for adenine (A) at nucleotide position1691 of FLV gene, which is located in exon 10. Thus, it results in the exchange of arginine (Arg) for glutamine (Gln) at position 506 of the protein, one of the main cleavage sites for protein C activation[16]. Consequently, this site has two alleles: allele G or non-mutant allele and allele A or mutant allele. The resistance to activated protein C, which is caused by the loss of FVL cleavage, generates hyper coagulopathy, hence increasing the risk to venous thrombosis[4]. The hereditary activated protein C resistance has been regarded as the main cause of most venous thrombosis cases. Moreover, 95% of APCR are associated with G1691A mutation in exon 10 of factor V gene[2, 6].

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Prothrombin or coagulation factor II is a blood protein synthesized in the liver in the presence of vitamin K. Moreover, it is a thrombin precursor, which induces the formation of fibrin at the end of the coagulation cascade. It is also involved in coagulation control mechanisms, binding to thrombomodulin and activating protein C, which also plays a fundamental role in anticoagulant balance[20]. The gene responsible for prothrombin contains approximately 21,000 pb, including 14 exons and 13 introns, located near the cetromere on chromosome 11[29].

In 1996, a mutation in the region 3' of the prothrombin gene was described (G20210A), which is closely associated with high prothrombin levels in the blood. Therefore, it is reported as medium risk to the formation of venous thrombosis. This allelic variant comprises a point mutation in which G replaces nitrogenous base A at nucleotide 20210 near the cleavage site of messenger ribonucleic acid precursor (mRNA) where the poly-A tail is added. This mutation is associated with a rise in mRNA stability and plasma concentration of prothrombin, which seems to be the mechanism that predisposes to the occurrence of thrombosis[20].

FVL mutation is present in several Caucasian populations and the prevalence of the heterozygous genotype varies from 2% to 13%. Furthermore, it is extremely rare among Africans, Chinese, Japanese, native Americans and South Asians[3]. The mutation in the prothrombin gene affects 1% to 3% of the Caucasian population, with a 2.8 fold higher risk of thrombosis. This mutation has not been detected among Afro-descendant or Asian patients to date, which indicates that in these groups the mutation is infrequent or extremely rare[1, 11, 24, 29]. Thus, this study had the objective to identify and estimate the frequency of three possible genotypes as well as the frequency of the two alleles present in factor V Leiden and prothrombin genes among patients with suspected thrombophilia in the state of São Paulo. This investigation may provide further literature and reference data on the incidence of prothrombin genotypes among individuals in Brazil.

MATERIAL AND METHODS

Material collection

In the present investigation, samples of peripheral blood were collected and sent to laboratories under contract with Genolab (Blumenau-SC), located in the state of São Paulo. The samples were collected according to the following protocol: samples kept at 4ºC for up to three days; no orientation as to patient's preparation; 5 ml of peripheral blood collected in tubes containing ethylenediaminetetraacetic acid (EDTA).

Deoxyribonucleic acid (DNA) extraction and amplification

We applied the phenol-chloroform extraction method for the isolation of DNA[23]. For the amplification of researched genetic material, we applied real time polymerase chain reaction (RT-PCR) with Taqman® probe and reagent concentrations were according to the technical specifications (Applied, Foster City, CA).

RT-PCR is a method that allows the quantification of genetic amplification products with the follow-up of PCR reaction in all phases and cycles. Accordingly, it dispenses with the use of conventional electrophoresis, optimizing the process[8]. The advantage of RT-PCR relies on the fact that it offers a higher sensibility when compared with conventional PCR, allowing the detection of low concentrations of genetic material in the sample[8].

Report analysis

We analyzed the results from exam reports on FLV and prothrombin genes from Genolab database from February 2008 to March 2012. The sample comprised 100 individuals with suspected thrombophilia. As the Brazilian population is highly mixed, there was no separation into ethnic groups.

After evaluating the reports, the genotypic and allelic frequencies were assessed.

RESULTS

10% of the patients from the total sample were male, whereas 90% were female. The higher number of exams among female patients is due to the fact that thrombophilia is associated with gestation problems, including miscarriages in the first trimester[19].

94% of the total individuals presented homozygous genotype G. There was no individual for homozygous genotype A and 6% presented heterozygous genotype for FLV gene. As to prothrombin gene, 97% had homozygous genotype G. There was no individual with homozygous genotype A and 3% had heterozygous genotype (**Table 1**).

TABLE 1 – Genotypic frequency of FVL and prothrombin genotypes in the studied populations

	FVL (%)	Prothrombin (%)
Homozygous G	94	97
Heterozygous	6	3
Homozygous A	0	0

FVL: factor V Leiden.

As to allelic frequencies, the following results were yielded: 97% for allele G and 3% for allele A in the FLV gene; 98.5% for allele G and 1.5% for allele A in the prothrombin gene (**Table 2**).

TABLE 2 – Allelic frequency of FVL and prothrombin alleles in the studied population

	FVL (%)	Prothrombin (%)
Allele G	97	98.5
Allele A	3	1.5

FVL: factor V Leiden.

DISCUSSION

In a study developed in Pernambuco, 39 individuals with mutation (13.3%) were detected[21]. Similarly, our research demonstrated an expected frequency in Caucasians, despite the fact that our population is ethnically mixed. In comparison with the investigation from Ramos *et al.*[21], another study also conducted in Pernambuco showed a frequency of 17.8% for the heterozygous genotype and 1.7% for homozygous A[17]. This variation may have occurred due to the fact that the researchers used PCR method followed by restriction fragment length polymorphism RFLP-PCR. Oliveira Filho *et al.*[17] applied RT-PCR, which is admittedly more sensitive than the previous one[13]. Nevertheless, Oliveira Filho *et al.* compared the efficacy of both techniques (RFLP-PCR and RT-PCR) and the results did not provide any significant difference.

A study developed by Soares *et al.*[26], in Minas Gerais, demonstrated a heterozygous rate of 1.9%. Another one conducted by Silva Filho *et al.*[25] revealed a rate of 0.7% and 3.6% in the population of Rio de Janeiro. Another research carried out by Palomo *et al.*[18] with the population from the central-south region of Chile showed a mutation rate of 1.25%. Accordingly, these studies presented a much lower rate in comparison with our results, which revealed a rate of 6% for the detection of heterozygous individuals.

As to the mutation in the prothrombin gene, in the study developed in Pernambuco, the genotypic frequency for the heterozygous genotype was 6%[22], whereas herein we demonstrated a higher frequency for the same genotype. The research carried out in the central-south region of Chile with a non-native population showed a frequency of 1.33% for the same genotype[18], thus a much lower frequency in comparison with our results. Another investigation carried out in Belém-PA demonstrated a frequency of 2% for heterozygous genotype[28], relatively similar to the results demonstrated herein.

Another investigation carried out with the general population and cases of severe widespread thrombosis in Mediterranean Spain revealed a frequency of 5.3% for heterozygous genotype in both populations[7]. The same investigation conducted in Minas Gerais yielded a frequency of 5.9% for heterozygous genotype[9]. Thus, in comparison with these studies, the population of São Paulo produced very similar results for the frequency of heterozygous genotype in the FLV gene.

The mutation in the prothrombin gene yielded a frequency of 3% for heterozygous genotype. This mutation is more commonly detected in the Caucasian population and it has not been found among Afro-descendant and Asian patients[11, 29].

In the study developed in Pernambuco, the genotypic frequency for the heterozygous genotype was 6%, contrasting with a higher frequency for the same genotype in the sample from São Paulo. The same study conducted in the central-south region of Chile with a non-native population showed a frequency of 1.33% for the same genotype[18], hence a much lower frequency in comparison with our results.

The investigation conducted in Belém-PA demonstrated a frequency of 2% for heterozygous genotype[28]. In contrast, the present study showed a relatively higher frequency. The study conducted with the general population and cases of severe widespread thrombosis in Mediterranean Spain showed a frequency of 5.3% for heterozygous genotype in both populations[7]. The same study conducted in Minas Gerais showed a frequency of 5.9% for the heterozygous genotype. Consequently, in comparison with these studies, the population of São Paulo presented lower results in the frequency of heterozygous genotype in the prothrombin gene.

In a study developed by Herkenhoff *et al.*[10], the frequency of heterozygous genotype was 7.26% in the state of Paraná and 15.13% in the state of Santa Catarina, respectively. The results yielded herein were lower in comparison with those from Paraná and significantly lower in comparison with those from Santa Catarina. As the south of Brazil was historically colonized by Europeans, mainly from Italian and German origins, it was expected that this investigation revealed a lower heterozygous genotype frequency as to the prothrombin gene. Furthermore, all studies mentioned above, with exception of that conducted by Herkenhoff *et al.*[10], applied RFLP-PCR method, with lower sensitivity when compared with RT-PCR. This may account for the low frequency of heterozygous genotype in the studies previously mentioned.

CONCLUSION

RT-PCR has a higher sensitivity in relation to other techniques applied in this study, resulting in different heterozygous genotype

figures. Therefore, it is recommended for the identification of point mutations or single nucleotide polymorphism (SNP).

Due to the fact that the literature does not provide enough information on this mutation in Brazil, these data will become a research tool for future reference, contributing to the database in Brazil.

The present investigation validated that the identification of this genotype is advisable for patients with suspected or confirmed thrombophilia, mainly considering the fact that our results substantiate a considerable frequency of mutations in the studied population.

RESUMO

Introdução: *A protrombina (fator II) é a precursora da trombina, que induz a formação de fibrina. Foi descrita uma mutação no gene da protrombina (G20210A), associado diretamente a altos níveis de protrombina no sangue e, consequentemente, a trombofilia. A mutação G1691A no gene do fator V de Leiden (FLV) localiza-se no éxon 10, resultando na alteração da proteína, um dos principais sítios de clivagem para ativação da proteína C.* ***Objetivos:*** *Identificar e estimar a frequência genotípica dos três possíveis genótipos, assim como estimar a frequência dos dois alelos existentes no gene do FLV e na protrombina em pacientes com suspeita de trombofilia no estado de São Paulo. Este estudo poderá fornecer mais dados para a literatura e para consulta da incidência dos genótipos da protrombina em indivíduos no Brasil.* ***Material e métodos:*** *Análise de mutação pontual por reação em cadeia da polimerase em tempo real (RT-PCR).* ***Resultado:*** *Obtivemos o número de 100 indivíduos e, desse total, 94% possuíam o genótipo para homozigoto G; apenas 6%, genótipo heterozigoto; nenhum indivíduo foi encontrado com genótipo homozigoto A no gene do FLV. No gene da protrombina, a frequência foi de 97% para o genótipo homozigoto G e 3% para o genoma heterozigoto; não foi encontrado nenhum indivíduo com o genoma homozigoto A.* ***Conclusão:*** *Este estudo mostrou que é recomendável a identificação do genótipo para esses genes em pacientes com suspeita de trombofilia nessa região.*

Unitermos: *fator V de Leiden; mutação g1691A; protrombina; mutação G20210A; PCR em tempo real; SNP.*

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