Blood Cells, Molecules and Diseases 66 (2017) 31–36

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Association of genetic polymorphisms with plasma TFPI level: Boon or curse for DVT patients – Study from India

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ARTICLE INFO

Editor: Mohandas Narla

Keywords:
Deep vein thrombosis
Tissue factor pathway inhibitor
Polymorphisms

ABSTRACT

Low plasma TFPI levels have been associated with an increased risk of DVT; however its association with TFPI gene polymorphisms is controversial and not yet studied in India. The aim of our study was to analyze prevalence of TFPI gene polymorphisms, evaluate their effects on its plasma levels and determine its association with DVT. Plasma level and genetic polymorphisms (33T > C, 399C > T and 536C > T) of TFPI were screened in subjects (100 DVT patients and 100 controls). Mean TFPI level in patients was significantly lower than controls (Patients: 33.55 ± 11.72 ng/ml, Controls: 48.05 ± 13.68 ng/ml, $p < 0.001$). DVT patients had significantly higher prevalence of 399C > T ($p = 0.001$, OR^a: 5.69, CI: 1.14–28.46) and lower prevalence of 33T > C polymorphism ($p < 0.001$, OR^a: 0.239, CI: 0.065–0.871). The wild type (TT genotype) of 33T > C and variant form (CT and TT genotype) of 399C > T polymorphism was significantly associated with low TFPI levels. TFPI 536C > T polymorphism was absent in all subjects. In conclusion, dual nature of TFPI gene polymorphisms were established in our association study; 33T > C being protective and 399C > T as an important risk factor in Indian DVT patients, probably mediated by alteration in TFPI levels. These findings may prove a vital role in risk stratification and treatment of DVT.

1. Introduction

Deep vein thrombosis (DVT) is a multifactorial disease and of great clinical significance because it is frequent, often associated with secondary venous dysfunction and it may lead to life threatening complications i.e. pulmonary thromboembolism [1–2]. Its risk factors may be classified into genetic and acquired. Genetic factors are deficiency of the coagulation inhibitors i.e. antithrombin (AT), protein C (PC) and protein S (PS) and the presence of mutations in the coagulation factors i.e. factor V Leiden mutation and prothrombin G20210A mutation [3–4]. Acquired factors include immobilization, surgery, trauma, pregnancy, malignant disease and antiphospholipid antibodies [1,5]. However no underlying genetic risk factor can be identified in about 30% of patients with a family history of DVT [6].

Tissue factor pathway inhibitor (TFPI) is a circulating, multidomain and Kunitz-type protease inhibitor. TFPI plays a major role to control the coagulation via inhibition of tissue factor–factor VIIa (FVIIa-TF) proteolytic activity. When the vessel wall is damaged, the exposed tissue factor (TF) initiates coagulation via FVII. TFPI reversibly binds and inactivates FXa and the TFPI-Xa complex inhibits FVIIa-TF complexes [7]. The human TFPI gene, which spans 70 kb, has been mapped to the chromosome 2 region q31–q32.1. It contains 9 exons and 8 introns [8].

Low levels of plasma TFPI have recently been shown to be associated with an increased risk of both venous [9] and arterial thrombosis [10,13]. These results are further supported by Kasthuri RS et al. who reviewed that reduced levels of TFPI are a risk factor for development of DVT [11]. Mechanisms involved in the regulation of plasma TFPI levels are not fully elucidated till now. Results from a recent genome-wide screening suggested that several single nucleotide polymorphisms of TFPI gene may be major determinants for variation in TFPI levels [12]. Further Opstad TB et al. in 2010 suggested that TFPI polymorphisms modulate its plasma levels [13]. Other studies have shown inconsistent results about the association between genotype and plasma levels of TFPI and its association with thrombosis [14–17].

The association between TFPI gene polymorphisms and their plasma

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http://dx.doi.org/10.1016/j.bcmd.2017.08.003
Received 19 May 2017; Received in revised form 4 August 2017; Accepted 4 August 2017
Available online 06 August 2017
1079-9796/

levels is not fully understood as per literature. Furthermore, the distribution of these genetic polymorphisms in the Asian population, particularly in India, has yet to be ascertained. Therefore, the aim of this study was to analyze the distribution of three TFPI polymorphisms (33T > C, 399C > T and 536C > T) in India, to evaluate the effects of these on plasma TFPI levels and to determine the relationship between TFPI and the development of DVT.

2. Methods

2.1. Subjects

A total of 100 young (age: < 45 years) patients with unprovoked DVT were recruited for this prospective case-control study at the Department of Hematology, All India Institute of Medical Sciences, New Delhi during year 2012–2015. DVT was diagnosed by compression ultrasonography and magnetic resonance imaging. All the patients enrolled in the study were screened for the genetic abnormalities like Factor V Leiden, heterozygous Protein S and C deficiency and the patients found negative for these investigations were included in the study. The patients were on anticoagulants for the duration of 1–3 years. Blood sample was collected at least 12 weeks after the discontinuation of oral anticoagulants. Patients receiving thrombolytic or anticoagulant treatment and hormonal therapy/oral contraceptive pills were excluded from this study. Patients with malignancy, pregnancy, surgery, liver disease and acute thrombotic phase were also excluded from the study. The patients enrolled in this case-control study were from different geographic region of India. 100 age and sex matched healthy controls who were non-blood related to the patients were taken for the study. The control subjects had no medical history of thrombosis. Written informed consent was obtained from both patients and controls. This study was granted ethical approval from AIIMS, institute ethics committee.

2.2. Laboratory investigation

5 ml venous blood was collected in 3.2% trisodium citrate solution and plasma was separated by centrifugation for 10 min at 3500 RPM at room temperature and stored at −70 °C. The platelet counts were performed on the samples after standard centrifugation and it was confirmed that the counts were minimal. Then these platelet poor plasma samples were further processed for TFPI levels by ELISA. We have not performed any test for platelet granule release during plasma preparation due to non-availability of the necessary equipments Plasma levels of TFPI-1 were determined by solid phase sandwich enzyme linked immunosorbent assay (ELISA) (Quatikine R & D Systems, Inc. USA).

Genomic DNA was isolated from whole blood using Bioserve DNA isolation kit. 536C > T and 33T > C polymorphisms were detected by PCR-RFLP. For 536C > T polymorphism, 170 bp fragment was amplified using the following primers: sense 5′TCTATTTTAATTGGCTGTAT-3′ and antisense- 5′GCATGATAA TAGTTTCCTGG-3′. This was followed by restriction digestion using BSrI enzyme (NEB) at 37 °C for overnight which yielded two DNA fragments when T allele was present (143 and 27 bp) and one fragment when C allele was present (170 bp). For 33T > C polymorphism, 402 bp fragment was amplified using the following primers: Sense 5′-CTCAATATACTGGACACATGGT-3′ and Antisense 5′-CAATTAATG GGAAGAGTATCTTC-3′. This was followed by restriction digestion using NdeI enzyme (NEB) at 37 °C for overnight resulting in yield of two DNA fragments when C allele was present (240 and 162 bp) and one fragment when T allele was present (402 bp) (Fig. 1).

For 399C > T polymorphism, allele specific PCR (ASP) was used to generate a PCR fragment of 179 bp (Fig. 2). The reaction was carried out in two separate tubes; one tube for wild type using forward primer 5′-GTTGGAGGTCTCTCTTAGTGTA-3′, and common reverse primer 5′-ACTTACATCAAGCCCAGAAA-3′ and second tube for the amplification of mutant type using mutant type forward primer 5′-GTTGGAGGTCT-CTCTTAGTGTG-3′ and same reverse primer.

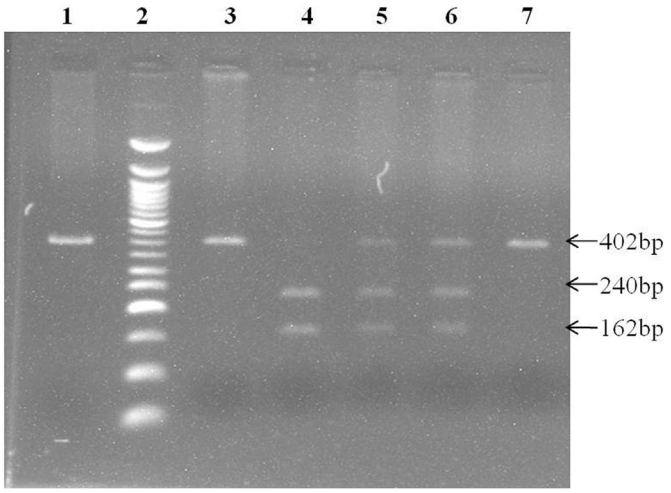

Fig. 1. 33T > C polymorphism: It was detected on 2.5% agarose gel. Explanation for the Lanes is as follows; Lane 1: undigested PCR product, Lane 2: 50 bp ladder, Lane 3 & 7: wild type, Lane 4: homozygous, Lane 5 & 6: heterozygous.

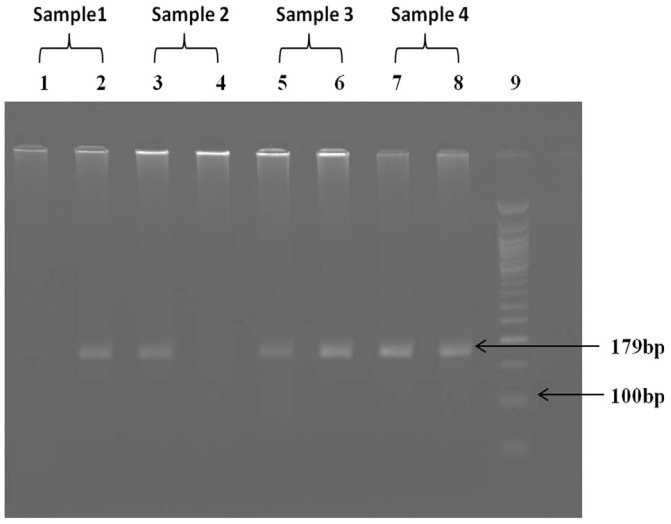

Fig. 2. 399C > T polymorphism (two tubes AS-PCR): It was detected on 2.5% agarose gel. Explanation for the Lanes is as follows; Lane 1 & 2 (sample 1): homozygous, Lane 3 & 4 (sample 2): wild, Lane 5–8 (sample 3, 4): heterozygous, Lane 9: 50 bp ladder.

2.3. Statistical analysis

Statistical analysis was performed by Stata 11. p values were 2 tailed, and statistical significance was set at $p < 0.05$. Comparison between categorical variables was performed using chi-square test and Fisher exact test was applied where value was < 5. Student *t*-test was used for the comparison of continuous variables. Allele frequencies were derived from genotypic data. Kruskal-Wallis test was applied to find out the association between genotype and their respective phenotype. Odds ratios (ORs) for DVT associated with TFPI polymorphisms and corresponding 95% confidence intervals (CIs) were calculated using logistic regression, adjusted for body mass index (BMI), Type II diabetes mellitus (DM), alcoholic and smoking status.