

A STUDY ON THE ROLE OF THROMBOPHILIC GENETIC DISORDERS AS A RISK FACTOR FOR THROMBOTIC COMPLICATIONS IN PATIENTS WITH MYELOPROLIFERATIVE DISORDERS

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Summary

Myeloproliferative neoplasms (MPN) are haematological diseases, characterized by clonal hematopoiesis. Hemostasis abnormalities are among the most critical and frequent complications, affecting the quality of life and a possible reason for death. Thrombotic complications are common and multifactorial. Our aim was to study some genetic thrombophilia factors – Factor V Leiden (FVL), G20210A mutation in prothrombin gene (PR G20210A) and PLA2 allele polymorphism of glycoprotein IIIa gene (GPIIIa gene), and their frequency and association with thrombotic risk in both Philadelphia-positive and Philadelphia-negative MPN – chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary and secondary myelofibrosis (MF). In our patient population, PLA2 allele polymorphism of GPIIIa gene proved to be the most common and significantly associated with thrombotic complications – 26.85% of our patients were carriers, and 24.14% of them reported thrombotic complications.

Key words: genetic thrombophilia, myeloproliferative neoplasms, thrombotic complications

Introduction

Myeloproliferative neoplasms (MPN) are clonal disorders of hematopoiesis, originating from stem cells. They are characterized by the accumulation of cells of different cell lines in the bone marrow, peripheral blood and spleen, as well as in other organs, that are prone to terminal differentiation [1]. The term covers chronic myelogenous leukaemia (CML), myelofibrosis (MF), polycythemia vera (PV), essential thrombocythemia (ET), as well as some rare conditions like chronic eosinophilic leukaemia, mastocytosis and others [2].

These conditions are divided into two main groups: Philadelphia-positive and negative, depending on Philadelphia chromosome carrier - t(9;22) (q34;q11). CML is Philadelphia-positive, while PV, ET and myelofibrosis (MF, primary and secondary) are Philadelphia-negative. The aetiology of these diseases is still not clearly defined, but many studies have determined the main pathogenic changes. Accumulation of terminally differentiated cells displaces normal hematopoiesis (clonality), resulting

from intracellular and intercellular signal pathways hyperactivation.

An essential step in CML pathogenesis is the presence of the Philadelphia chromosome, leading to BCR-ABL fusion gene formation. It produces a continuously active protein that activates molecular cascade, accelerating cell division. In Philadelphia-negative MPN (PV, ET and MF) JAK-STAT signal pathway plays a primary role – a point mutation JAK2 V617F (9p24) leads to increased signals, independent of physiological stimuli, from the cell surface receptors to the nucleus.

Hemostasis disorders in Philadelphia-negative MPN are one of the main pathogenic mechanisms, leading to late complications and even death [1]. Hemostasis is of the procoagulant type. Thrombotic complications are not that significant in CML, and the thrombophilic carrier is slightly studied [3-6].

Thrombosis pathogenesis includes different factors:

- Erythrocytosis – high hematocrit levels in patients with PV lead to blood hyperviscosity and blood stasis (especially in the brain);
- Thrombocytosis;
- Functional and structural platelet abnormalities;
- Platelet membrane receptors – increased GP Ib, GP IIb/IIIa receptors;
- Leukocytosis and leucocyte activation – increased level of platelet-leucocyte aggregates (CD11b/CD18 expression);
- Endothelium dysfunction;
- Age – above 60 years;
- History of thrombotic complications;
- Cardiovascular diseases and other potent thrombogenic factors;
- Smoking;
- Inherited and acquired thrombophilia and others [7].
- Thrombophilia is the predisposition to venous and arterial thrombosis. Inherited and acquired conditions may cause abnormal coagulation. Amongst the essential thrombophilia genetic factors as a cause for thrombosis are:
 - Factor V Leiden (FVL) – point mutation that leads to activated protein C inability to recognize its factor V binding place. This results in uncontrolled prothrombin activation;
 - G20210A mutation in the prothrombin gene (PR G20210A) that leads to prothrombin hyperproduction;
 - Platelet glycoprotein IIb/IIIa (GLPR IIb/IIIa – PLA2/GPIIIa) is a fibrinogen receptor; a place where fibrinogen binds von Willebrand factor on the platelet surface. Carrier of this mutation is associated with increased platelet aggregation [8-10] and thrombosis risk.
 - These mutations exert their influence on physiological hemostasis through different mechanisms:
 - FVL – a point mutation, where guanine is replaced by adenine at nucleotide 1691, leading to the formation of glutamine instead of arginine at amino acid 506 in factor V amino acid chain. It is one of the three binding points for activated protein C to factor V heavy chain. Activated factor V is a cofactor in the prothrombinase complex, leading to the formation of thrombin. Activated protein C degrades factor VIIIa. In the presence of this point mutation, the abnormal factor V is resistant to proteolytic inactivation of activated protein C, and protein S. FVL remains procoagulant and leads to hypercoagulation;
 - Moreover, FVL is an abnormal cofactor for activated protein C when degrading factor VIIIa. Thrombin activatable fibrinolysis inhibitor is also activated, leading to thrombin generation. TAFI is a fibrinolysis inhibitor, and this explains why the thrombus is resistant to fibrinolysis;
 - PR G20210A – a substitution of guanine with adenine at 20210 nucleotide of the prothrombin gene. Thrombin plays a central role in physiological hemostasis. It leads to the transformation of fibrinogen to fibrin, activates other coagulation factors and platelets. Prothrombin is increased when this mutation is present. The exact thrombogenic mechanism is not clear yet;
 - GPIIIa gene – this platelet receptor for fibrinogen is a common pathway in platelet activation and is formed by two subunits (GPIIb/IIIa). It plays an essential role in platelet aggregation. Polymorphism is a point mutation, in which cytosine is replaced by thymidine in exon 2, phenotypically

leading to the replacement of proline with leucine in 33 position of GPIIIa [11, 12].

- Different guidelines present recommendations for patient testing when thrombophilia carriership status is suspected [13-15]. Knowledge about many factors may be necessary for the prognosis and therapy optimization – genetic thrombophilia, JAK2 V617F mutation, CD11b/CD18 expression, comorbidities.

We aimed to study the role of some genetic thrombophilia factors (FVL, PR G20210A, and GLPR IIb/IIIa – P1A2/GPIIIa) in thrombogenesis of MPN patients. An objective of this article was to determine the frequency of genetic thrombophilia carriership status in MPN patients (isolated and combined) and compare it with the control group. We also aimed to determine the carriership frequency in patients with thrombotic complications with or without a family history, and patients without such complications. Another objective was to investigate the association between thrombophilia carriership status and thrombosis risk.

Materials and Methods

We used selective screening to determine MPN patients. Informed consent was obtained from all patients, as well as information about passport data, disease history and treatment, comorbidities (myocardial infarction, ischemic heart disease, arterial hypertension, heart failure, diabetes, obesity, hyperlipidemia, liver conditions, and neoplasms). Special attention was paid to questions about thrombotic complications before and after diagnosis, family history and most common thrombosis provoking factors – surgery, trauma, prolonged immobilization, malignancies, hormone therapy, and smoking. In women, we inquired about spontaneous abortions and pregnancy complications. Seven to ten millilitres of venous blood was obtained.

We performed deoxyribonucleic acid (DNA) extraction. Genomic DNA extraction from peripheral blood leucocytes was performed using the AccuPrepR Genomic DNA Extraction Kit (Bioneer, South Korea), according to the manufacturer's instructions, or by the salting-out method. Spectrophotometric analysis was used to determine the purity and the concentration of

the isolated samples. All isolated genomic DNA was stored at -20°C until further analysis.

FVL genotype determination

To identify the presence of FVL G1691A polymorphism, a 267 bp region from exon 10 of the factor V gene was amplified by polymerase chain reaction (PCR) using the following primer set: forward primer 5'-TGCCCAGTGCTTAACAAGACCA-3'; reverse primer 5'-TGTTATCACACTGGTGCTAA-3' [16]. The PCR amplification was performed with an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 54.8°C for 30 s and 72°C for 50 s, and a final extension step of 72°C for 10 min. After that, 5 µL of the amplified product was digested with 10U Mnl I restriction enzyme at 37°C overnight, according to the relevant manufacturer's instructions. The digested fragments were separated on a 2.5 % agarose gel, containing 50 µg/mL ethidium bromide, for 1 h at 120 V and were visualized under UV light. Digestion of the wild-type allele with Mnl I resulted in the generation of three fragments of 163 bp, 67 bp and 37 bp, whereas two fragments of 200 bp and 67 bp were produced upon digestion of the FVL G1691A allele [16].

Prothrombingenotypedetermination

G20210A polymorphism of the gene encoding prothrombin was detected using PCR-restriction fragment length polymorphism (RFLP) analysis. A 345 bp fragment in the prothrombin gene was amplified using specific oligonucleotide primer set: forward primer 5'-TCTAGAAACAGTTGCCTGGC-3'; reverse primer 5'-ATAGCACTGGGAGCATTGAA-3' (2). The DNA was denatured at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 54.8°C for 30 s and 72°C for 50 s, and a final extension step of 72°C for 10 min. Then, 5 µL of the amplified product was digested with 10U Hind III restriction enzyme at 37°C overnight, according to the relevant manufacturer's instructions. The digested fragments were separated on a 2.5 % agarose gel containing 50 µg/mL ethidium bromide for 1 h at 120 V and visualized under UV light. Digestion of the wild-type allele with Hind III resulted in an undigested fragment of 345 bp, while the mutant G20210A allele gave

two fragments of 322 bp and 23 bp [17].

GPIIb/IIIa genotype determination

Polymorphism PLA1/A2 in the gene for GPIIb/IIIa was determined by allele-specific restriction enzyme analysis. A 266 bp sequence comprising exon 2 of the GPIIIa gene was amplified by polymerase chain reaction using specific oligonucleotide primer set: forward primer 5'-TTCTGATTGCTGGACTTCTCTT -3'; reverse primer 5'-TCTCTCCCCATGGCAAA GAGT -3' [18]. After an initial 10-minute denaturation at 95°C, 45 temperature cycles were carried out consisting of 30 s at 95°C, 30 s at 54.8°C, and 50 s at 72°C, followed by a final extension step of 10 min at 72°C. Next, 5 µL of the amplified product was digested with 10U Msp I restriction enzyme at 37°C overnight, according to the relevant manufacturer's instructions. The digested fragments were separated on a 2.5 % agarose gel containing 50 µg/mL ethidium bromide for 1 h at 120 V and visualized under UV light. Digestion of the wild-type PLA1 allele with Msp I resulted in the generation of two fragments of 221 bp and 45 bp, whereas three fragments of 171 bp, 50 bp and 45 bp were produced upon digestion of the PLA2 allele [19].

To determine statistical significance we used p-value.

We investigated 108 MPN patients, aged 23

to 83 years (mean value 62.03), hospitalized in Hematology Clinic or out-patients, at University Multiprofile Hospital for Active Treatment "Dr. Georgi Stranski" – Pleven, Bulgaria between March 2013 and March 2018. Sex distribution was 43 females and 65 males (1:1.5).

Control groups included 147 healthy volunteers without MPN diagnosis or thrombosis, average age 31.99 years, and the females: males ratio was 1:1.67.

Results

Thrombophilia FVL carriership status

We found 104 (96.30%) non-carriers and 4 (3.70%) FVL carriers (all heterozygous) – Figure 1. The carriers were 2 patients with PV, and 2 patients with CML. No thrombotic complications were registered in these patients. The frequency found in the control group was as follows: 140 (95.24%) non-carriers and 7 (4.76%) FVL carriers (all heterozygous).

PR G20210A carriership status

We found that 100 (92.59%) of our patients were non-carriers and 8 (7.41%) were heterozygous carriers. In the control group, 144 patients (97.96%), and 3 patients (2.04%) were heterozygous carriers (Figure 2). Chi-square

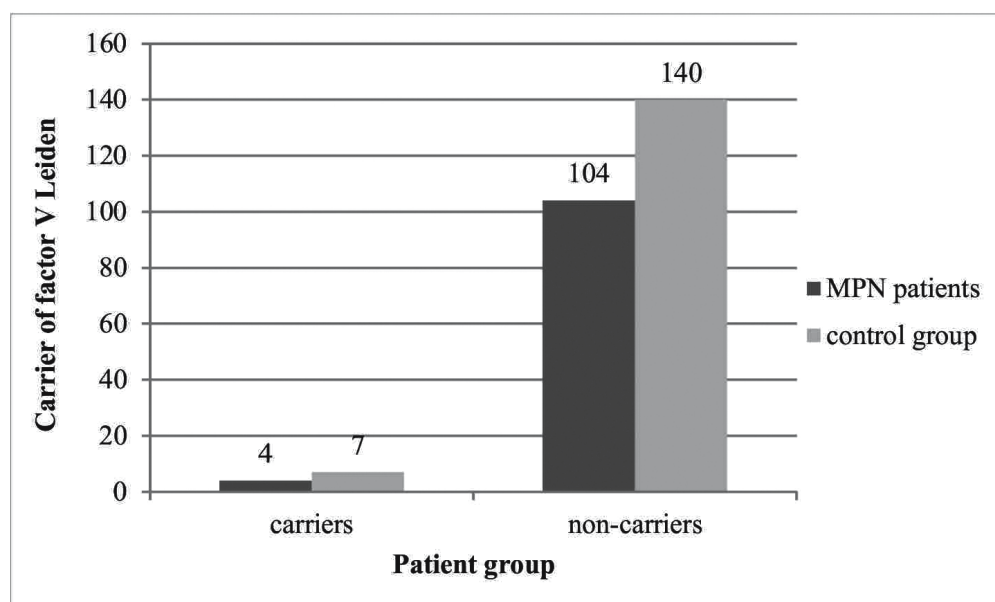


Figure 1. Distribution of MPN patients and control group according to factor V Leiden carriers (Number)

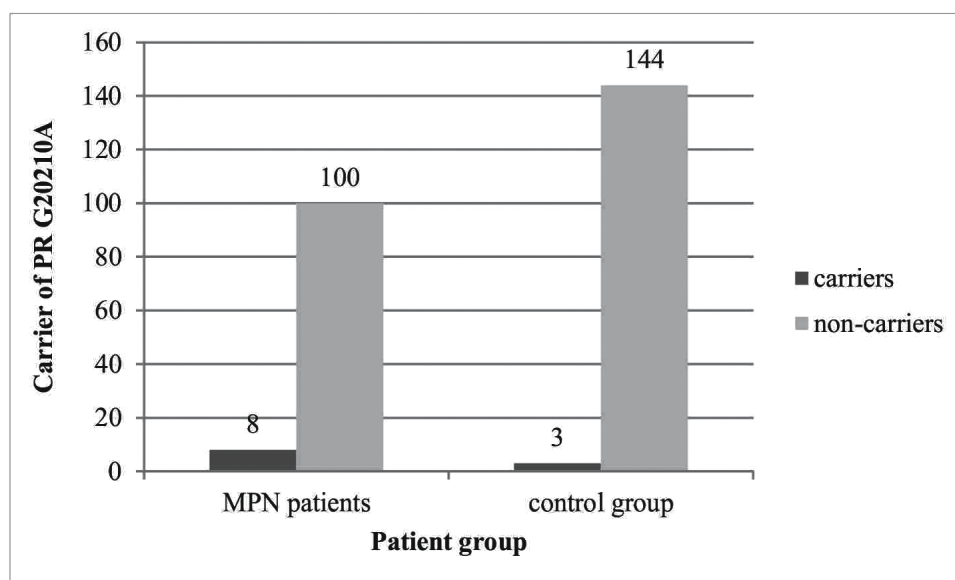


Figure 2. Distribution of MPN patients and control group according to PR G20210A carriers (Number)
 *95% CI – 0.1402 to 12.0397, Chi 4.332, $p = 0.037$

was 3.141, $p=0.076$, OR 3.840, RR 3.630. The carriers were 4 patients with MF, 3 patients with CML (one with thrombotic complication), and one - with PV.

Of the patients we investigated, two of the 8 PR G20210A carriers had a thrombotic complication, and there were no complications in the three control group carriers. RR 2.2222 ($p=0.576$), OR 2.0588 ($p=0.666$).

GPIIIa gene

We found 79 (73.15%) non-carriers, four (3.70%) homozygous carriers, and 25 (23.15%) heterozygous carriers, whereas in the control group there were 134 (91.16%) non-carriers, one (0.68%) homozygous and 12 (8.16%) heterozygous (Figure 3). Total mutation carriership in our patients was 26.85% (29 out of 108 patients), 8.84% (13 out of 147) in the control group, $p<0.001$ (95% CI 8.6254 to 27.7476, Chi 14.623). Homozygotes were found in two PV patients and two MF patients, heterozygotes – in 5 PV patients, 6 ET patients, 5 MF patients, and 9 CML patients. Six of the heterozygous carriers (2 PV patients, 2 CML patients, 1 ET and 1 MF patients) and one homozygous carrier (PV) reported thrombotic complications – 24.14% of PLA2 allele carriers reported thrombotic complications.

Combined carriership status

We found 3 (2.78%) patients with combined

carriership – two of them were G20210A and PLA2/GPIIIa heterozygous and one FVL and PLA2/GPIIIa heterozygous. Only one patient reported a thrombotic event. These data could not be conclusive.

Thrombotic events

Twenty-seven (25.00%) of our patients reported a past thrombotic event before diagnosed. Fifteen (13.89%) patients reported thrombotic complications after diagnosis: 6 (5.55%) reported ischemic brain attacks, 8 (7.41%) - deep vein thrombosis, and 2 (1.85%) - spleen infarction.

Thirty (27.78%) of our patients reported thrombotic event before or after diagnosis, and 12 (40.00%) of them – both. Eight of these 30 patients were found with thrombophilia mutations (Chi 0.357, $p=0.550$, OR 1.442, RR 1.319).

In 38 (35.19% of all patients) patients, we found thrombophilia carriership, and 8 (21.05%) of them reported thrombosis.

Discussion

FVL is an autosomal-dominant disease. It is reported to increase the risk of thrombosis 5-10 times in heterozygous carriers, and 50-100 times - in homozygous ones (as compared to non-carriers) [20-22]. The usual frequency of FVL is 2-15%, and up to 40% in patients with thrombotic complication [11, 23-25]. We did not

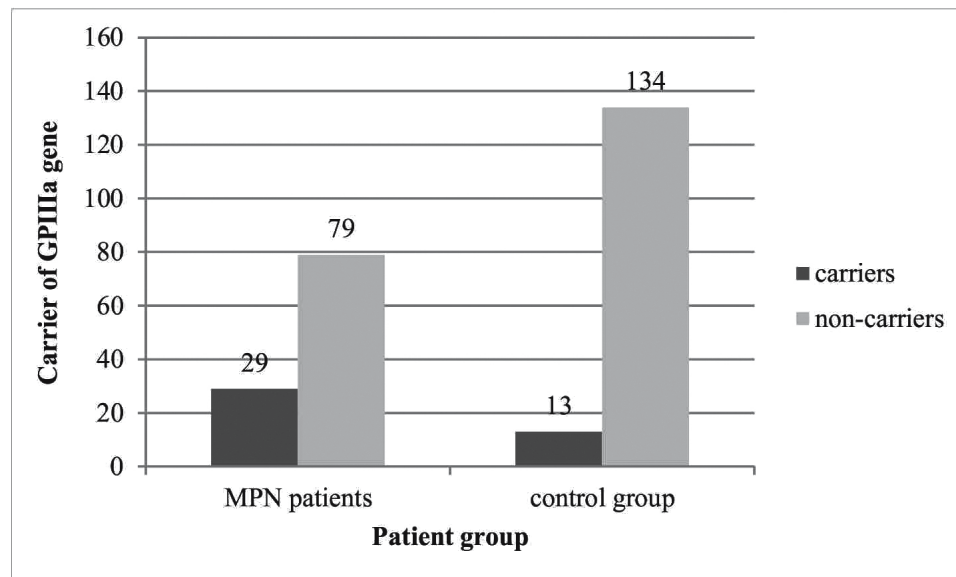


Figure 3. Distribution of MPN patients and control group according to PLA2 allele in GPIIIa gene carriers (Number)

* $p < 0.001$, 95% CI 8.6254 to 27.7476, Chi 14.623.

find any higher FVL frequency in our patient population, as compared to the control group (3.70% vs 4.76%). No higher thrombosis rate was found either. Jensen et al. (2002) reported significantly increased Factor V Leiden mutation prevalence in MPN patients versus controls (9% vs 3,4%; $p = 0.003$) [26]. Other authors have also confirmed a positive relationship between FVL and thrombosis in MPN patients [27, 28] and have emphasized on the fact that FVL carriership was associated with higher thrombosis relapse rate [28]. Other authors have reported data similar to ours: no significantly increased prevalence of thrombotic complications in FVL carriers with MPN diagnosis [29].

Prothrombin mutation (G20210A) is found in 1-2% of the population and up to 20% in patients with thrombosis [29, 30]. According to literature data, heterozygous carriers are exposed to 3-5 times higher thrombotic risk [21, 22, 30, 32, 33]. The total mutation frequency in our patients was 7.41% (8 out of 108 patients – all heterozygous) and 2.04% in control group (3 out of 147 healthy controls – all heterozygous), $p = 0.037$ (95% CI, 0.1402-12.0397, Chi 4.332). No significantly higher thrombotic risk was confirmed: RR 2.2222 ($p = 0.576$), OR 2.0588 ($p = 0.666$). This finding is also reported by several other studies [27, 31]. However, some authors have confirmed a higher thrombotic risk among MPN patients

with prothrombin mutation carriership [34].

Although GPIIIa polymorphism proved to activate platelets, no specific association exists between its carriership and thrombotic risk [35]. A meta-analysis of studies have confirmed its importance for coronary heart disease risk [36], but little data is published on its prevalence in MPN patients and contribution to thrombosis in these populations [29]. According to our results, this mutation is statistically significantly more frequent in our patients than in the control group. Of 29 GPIIIa carriers, 7 thrombotic complications were registered while in the control group no thrombosis was found in 13 carriers. RR 7.0000, OR 9.0000. Among GPIIIa carriers, 7 thrombotic events were registered in 108 patients (6.48%). These 7 patients represented 25.93% of all 27 MPN patients with thrombotic events. It was previously reported that PV patients with GPIIIa had increased thrombotic risk [29].

Most of the studies available on thrombophilia and thrombotic risk include patients, diagnosed with PV and ET, rarely MF and CML or all four of these.

Conclusions

Thrombophilic disorders play a role in thrombotic complications pathogenesis in MPN patients. To define its contribution, a complex study is

necessary on all possible risk factors, and their interpretation should be defined as a part of this multifactorial pathogenesis. This would lower the risk of a common and dangerous complication like thrombosis. We did not find a significant correlation between FVL and G20210A polymorphisms and increased thrombotic risk in MPN patients. Currently, few study reports exist on GPIIIa relation to thrombotic complications. In our patient population, this polymorphism appeared frequently and significantly increased the thrombotic risk. A more significant number of patients, as well as patients being prospectively investigated for yet unrevealed thrombosis, would confirm relevant results.

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