The Impact of JAK2 Mutation on Thrombosis in Philadelphia Chromosome-Negative Chronic Myeloproliferative Neoplasms

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ABSTRACT

Chronic myeloproliferative neoplasms (CMNs) are related to clonal over-proliferation of hematopoietic stem cells. Polycythemia vera,essential thrombocythemia and primary myelofibrosis are included in CMNs.An important complication of these diseases is thrombotic events. The relationship between Janus Kinase gene mutation and thrombosis in CMNs is still debated. Herein, we have aimed to investigate the impact of JAK2 mutation on thrombosis in philadelphia negative (Ph-) CMNs. The present study included 208 patients with Ph-CMNs who applied to the hematology clinic of Diskapi Yildirim Beyazit Training and Research Hospital for treatment and followed-up between 2013-2019. The patient data were retrospectively reviewed.Statistical analysis was performed with SPSS V.25.0. The statistical significance level was accepted as $p< 0.05$.Multivariate analysis was performed to the variables with $p< 0.01$ in the univariate analysis. When comparing the thrombosis and non-thrombosis groups in univariate logistic regression analysis, there was a borderline statistically significant difference in gender ($p= 0.058$) and statistically significant differences were found in the presence of diabetes mellitus ($p= 0.001$), hypertension ($p< 0.001$), hyperlipidemia ($p= 0.025$) and serum calcium ($p= 0.028$).According to multivariate logistic regression analysis,gender ($p= 0.032$), diabetes mellitus ($p= 0.027$),and serum calcium ($p= 0.031$) were found to be associated with thrombosis. When these three parameters were examined together, 94.2% sensitivity and 43.8% specificity were detected. The overall predictive accuracy for thrombosis was found to be 84.2%.Also there was statistically significant difference in sedimentation, C-reactive protein, AST levels and size of liver between the thrombotic and non-thrombotic patients in univariate analysis. There was no statistically significant relationship between the presence of JAK2 mutation ($p= 0.637$) and JAK2 allele burden ($p= 0.885$) with thrombosis. Cardiovascular risk factors were found to be related with thrombosis in Ph-CMNs rather than presence of JAK2 mutation or JAK2 allele burden.

Keywords: Polycytemia vera, Essential thrombosis, Primary myelofibrosis, JAK mutation, Thrombosis

INTRODUCTION

Chronic myeloproliferative neoplasms (CMNs) are a group of hematological diseases characterized by an increase in the number of mature blood cells in one or more lineages as a result of the excessive proliferation of hematopoietic stem cells in the bone marrow.[1] One of the diseases in this group is chronic myeloid leukemia (CML), which is characterized by an increase in mature blood cells in one or more lineages and the presence of the BCR-ABL fusion gene resulting from the t(9;22) translocation, also known as the Philadelphia chromosome. Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are other CMNs that are negative for the Philadelphia chromosome.[2] Thromboembolic events are a major cause of morbidity and mortality in this group of diseases, which result from various somatic mutations such as Janus kinase 2 (JAK2) mutations.[3,4] Ischemic stroke, acute myocardial infarction, unstable angina pectoris, and peripheral arterial thrombosis are common events.

** All of the authors were working at Diskapi Yildirim Beyazit Training and Research Hospital during the study. Most of the departments of the hospital has been closed at September 2022, and the authors of the papers are specified according to their current institutions.*

Additionally, cerebral and splanchnic venous thrombosis, deep vein thrombosis, transient ischemic attacks, and retinal artery or vein occlusion may also occur.[4] Risk factors for thrombosis in philadelphia negative (Ph-) CMNs have been defined as male gender, previous thrombosis, the presence of cardiovascular risk factors, leukocytosis, and JAK2 V617F mutation.[4] Nearly all PV patients have a positive JAK mutation. Therefore, the effect of the mutation on thrombosis has been evaluated more through mutation burden. While some studies suggest that as the mutation burden increases, the frequency of thromboembolic events increases, other studies conclude that the mutation is unrelated to thrombosis.[5,6] In ET and PMF, more than half of the patients have a positive JAK mutation. While some studies suggest that the mutation is unrelated to thrombotic propensity, generally observed data is consistent with the hypothesis that the presence of JAK mutation increases the frequency of thrombosis.[7,8,9] Thrombotic events such as acute coronary syndrome, transient ischemic attack, and ischemic stroke are serious causes of morbidity and mortality in BCR-ABL negative CMNs. Predicting thrombosis risk in these patients will result in more successful disease management. This study aims to investigate the impact of JAK2 mutation on thrombosis in Ph- CMNs.

PATIENTS AND METHOD

Two hundered and eight (208) patients who presented to the hematology clinic at Ankara Diskapi Yildirim Beyazit Training and Research Hospital from 2013 to 2019 and diagnosed as PMF, PV or ET were included in our study. Patients who were younger than 18 years and diagnosed as CML were excluded from the study. This study was designed as a retrospective observational study. Data were obtained from the hospital digital database and patients' hospital records. Of the 208 patients included, 12 were diagnosed with PMF, 104 with ET, and 92 with PV.

The study collected data including patient file numbers, genders, diagnoses, diagnosis dates, whether they had died and if so, the date and cause of death, treatments received, comorbidities, whether they developed thrombosis, bone marrow biopsy results for patients who underwent follow-up, results of genetic testing related to Ph- CMNs, smoking status, and last evaluation dates. The study analyzed various parameters including hemogram and biochemistry data at the time of diagnosis. The patients were divided into three groups based on their platelet count: less than 450 x 10^9/L, between 450 x 10^9/L and 1000 x 10^9/L, and above 1000 x 10^9/L. Patients with a liver size greater than 120 mm were considered to have hepatomegaly, while patients with a spleen size greater than 120 mm were considered to have splenomegaly. The study examined how many times thrombosis occurred in patients who developed thrombosis, the location of thrombosis, and whether there was any accompanying clinical condition that provoked thrombosis. In addition, hereditary thrombophilia panel, protein C, protein S, and antithrombin 3 levels, anticardiolipin antibody, lupus anticoagulant, antiphospholipid antibody, and anti-beta-2-glycoprotein1 results were also examined. The genetic analysis included JAK2 V617F mutation status, allele burden, and CALR gene mutation status. JAK2 mutation positive patients were divided into four groups based on their allele burden: 1-24%, 25-49%, 50-74%, and 75-100%. In addition, 13 patients were tested for the presence of exon 12 mutations, and all were found to be negative. MPL mutation was tested in 22 patients, including 2 with PMF and 20 with ET, and all were found to be negative.

Ethical Board Approval

This study has been accepted as internal medicine specialization thesis of Ayse Arslan Kapuci. Ethical approval was obtain from Ankara Diskapi Yildirim Beyazit Training and Research Hospital Ethical Committee (Date: 02.03.2020 / No: 83/17).

Statistical Analysis

All statistical analyses were performed using IBM© SPSS© Statistics Version 25.0 for Windows (Armonk, NY: IBM Corporation, 2017). Firstly, descriptive statistics were performed for patients. Categorical data were analyzed with chi-square or Fisher's exact test. The distribution of continuous

Table 1. Analysis of categorical data with the Chi-Square test

Parameters	No Thrombosis	Thrombosis Present	p
Gender (male/female)	78/86	28/16	0,058
Diagnosis (Polycythemia vera/ essential thrombocythemia/ Primary myelofibrosis)	74/79/11	18/25/1	0,398
JAK2 V617F mutation (absent/present)	32/132	10/34	0,637
JAK mutation allele burden (1-24% / 25-49% / 50-74% / 75-100%)	50/42/18/22	11/12/5/5	0,941
Exitus (absent/present)	154/10	39/5	0,231
Bone marrow fibrosis degree (Grade 1/2/3)	12/10/17	5/4/3	0,513
Liver and spleen size (normal/hepatomegaly/splenomegaly/ hepatosplenomegaly)	55/28/28/53	13/11/8/11	0,579
CALR mutation (absent/present)	13/8	4/1	0,628
Platelet count (<450x10^9/L -- 450x10^9-1000x10^9/L -- >1000x10^9/L)	41/95/27	10/25/8	0,936
Cytoreductive therapy (absent/present)	18/108	7/32	0,193
Smoking status (non-smoker/smoker)	131/33	38/6	0,328
Diabetes Mellitus (absent/present)	146/18	30/14	0,001
Essential Hypertension (absent/present)	102/62	14/30	<0,001
Hyperlipidemia (absent/present)	147/17	33/10	0,025

data was analyzed with the Kolmogorov-Smirnov test. Normally distributed continuous data were examined using t-tests or ANOVA. Non-normally distributed data were examined using the Mann-Whitney U test or Kruskal-Wallis test. Kaplan-Meier test was used for survival analysis. The statistical significance limit was set at $p< 0.05$. To measure the causality, the univariate logistic regression analysis was performed. Those with p-values less than 0.1 in the univariate analysis were included in the multivariate logistic regression analysis. The backward stepwise method was used for the multivariate logistic regression analysis.

RESULTS

The gender distribution of the 208 patients included in the study, consisting of 92 PV, 104 ET, and 12 PMF cases, was balanced. It was observed that 15 of the 208 patients had died. JAK2 V617F mutation analysis had been performed for all included patients. Among the 26 patients who underwent CALR mutation analysis, 9 had a positive result, and all 13 patients who were examined for Exon 12 mutation had a negative result. MPL mutation was examined in a total of 22 patients, and all were negative. There were 32 patients with diabetes mellitus (DM), 92 patients with hypertension (HT), and 27 patients with (hyperlipidemia) HL. Among the total, 140 patients were given cytoreductive treatment. The continuous data of the patients were compiled, and the median, minimum, and maximum values were determined and categorized. A total of 208 patients participated in the study, ranging in age from 20 to 91 years, with a median age of 64. The median absolute white blood cell count at diagnosis was 11,000 cells/mm^3. The median hemoglobin value at diagnosis was 15.4 g/dL, and the median hematocrit value was 47.5%. The median platelet count at diagnosis was 605500 cells/mL. Erythropoietin values ranged from 0.1 to 707 mIU/mL, with a median value of 3.09. The median homocysteine level was 22.5 mmol/L. The median LDL value was 112.5 mg/dL, the median HDL value was 40.5 mg/dL, the median triglyceride value was 131 mg/dL, and the median total cholesterol value was 174 mg/dL at diagnosis. After categorizing the data of the patients as categorical and continuous, the differences in categorical data between the groups with and without thrombosis were examined using the Chi-Square test (Table 1). There were no statistically significant differences between the groups with and without thrombo-

Table 2. Analysis of continuous data related to thrombosis using t-test

Parameters	No Thrombosis (median)	Thrombosis Present (median)	p-value
Age (years)	62	61	0.800
JAK mutation allele percentage (%)	24.5	26	0.826
Liver size (cm)	170	170	0.189
Spleen size (cm)	144	145	0.951
White Blood Cell Count (cells/mm^3)	11600	11400	0.503
Hemoglobin (g/dl)	15.4	14.8	0.847
Hematocrit (%)	48	45.9	0.921
Platelet (cells/ml)	597000	650000	0.140
Creatinine (mg/dl)	0.90	1.06	0.272
Urea (mg/dl)	35	33.5	0.707
ALT (U/L)	18	18	0.384
AST (U/L)	21	22	0.152
C-Reactive Protein (mg/L)	3.27	4.1	0.099
Sedimentation (mm/h)	7.0	15	0.033
Calcium (mg/dl)	9.6	9.26	0.027
Phosphorus (mg/dl)	3.48	3.43	0.750
Total Protein (g/dl)	7.4	7.2	0.082
Albumin (g/dl)	4.4	4.2	0.585
Uric Acid (mg/dl)	5.8	6.4	0.255
LDH (U/L)	260	281	0.834
Vitamin D (ng/ml)	15.3	14.4	0.506
Vitamin B12 (pg/ml)	285	281.5	0.208
Folate (ng/ml)	7.7	7.6	0.762
Erythropoietin (mIU/ml)	3.06	3.81	0.850
Homocysteine (mmol/L)	17.45	25.8	0.269
LDL (mg/dl)	113	111	0.696
HDL (mg/dl)	41	32	0.282
Total Cholesterol (mg/dl)	175	122	0.654
Triglycerides (mg/dl)	131	170	0.683

sis in terms of patient diagnoses, JAK2 mutation and mutation allele percentages, CALR mutation, presence of bone marrow fibrosis, hepatosplenomegaly, platelet counts, cytoreductive therapy, and smoking. When comparing the thrombosis and non-thrombosis groups, there was a statistically significant difference in gender (p= 0.058). When comparing the thrombosis and non-thrombosis groups, statistically significant differences were found in the presence of DM (p= 0.001), HT (p< 0.001), and HL (p= 0.025).

The continuous data related to thrombosis were analyzed by t-test, and sedimentation values showed a statistically significant difference between the thrombotic and non-thrombotic groups (p= 0.033). In addition, calcium values also had a statistically significant difference between the two groups (p= 0.027). C-reactive protein and total protein levels had a borderline statistical significance. No statistically significant difference was observed between the two groups for other parameters (Table 2). Logistic regression analyses were performed to investigate the effect of single variables on thrombosis. Coexisting DM, HT, HL, calcium, sedimentation rate, C-reactive protein, AST values at diagnosis, gender and liver size were found to be associated with thrombosis. Additionally, borderline statistical significance was found when examining the

Table 3. Multivariate logistic regression analysis backwards likelihood ratio

Parameters	B	Exp (B)	P value
Gender	1.486	4.419	0.032
DM -1.670	0.188	0.027	
Calcium	-1.312	0.269	0.031

Table 4. The relationship between JAK mutation, allele burden, and platelet count with thrombosis for patients with ET and PMF

Diagnosis	Parameters	No Thrombosis	Thrombosis Present	p
ET	JAK2 V617F mutation (absent/present)	28/51	10/15	0.812
	JAK mutation allele burden (1-24/25-49/50-74/75-100)	28/15/3/5	6/6/1/1	0.794
	Platelet count (<450x10^9/L/450x10^9/L-1000x10^9/L/>1000x10^9/L)	1/51/26	0/17/7	0.784
PMF	JAK2 V617F mutation (absent/present)	4/7	0/1	0.460
	JAK mutation allele burden (1-24/25-49/50-74/75-100)	0/1/3/3	0/0/1/0	0.565

relationship between total protein value and cytoreductive therapy with thrombosis. The patient's specific MPN diagnosis, JAK mutation, allelic burden, CALR mutation, smoking status, degree of fibrosis in the bone marrow, white blood cell and platelet counts at diagnosis, hemoglobin, hematocrit, erythropoietin, and homocysteine levels were found to be unrelated to thrombosis. The significant parameters found in the univariate logistic regression analysis were examined with multivariate logistic regression analysis backwards likelihood ratio. Gender, DM, and calcium values at the time of diagnosis were found to be associated with thrombosis (Table 3). When these three parameters were examined together, a sensitivity of 94.2% and a specificity of 43.8% were detected. The overall predictive accuracy for thrombosis was found to be 84.2%.

The average overall survival was found to be 5293 days in patients who did not have thrombosis, and 7463 days in patients who did have thrombosis which results in no statistically significant difference (p= 0.082). JAK mutation and allele burden were evaluated by chi-square test for patients with ET and PMF after all these analyses. As in all patients, the relationship between these parameters and thrombosis was not detected in these groups. The data are presented in Table 4. In addition, the relationship between the number of thromboses that patients experienced and JAK mutation and allele burden was also examined. A statistically significant result was not found (Table 5).

DISCUSSION

Thrombosis is an important factor affecting both morbidity and mortality in Ph- classical CMNs.[4] It is believed that hyperviscosity in polycythemia vera creates a tendency for thrombosis.[10] In addition, it is known that factors such as advanced age, leukocytosis, and a high allele burden of the JAK2 V617F mutation are risk factors for thrombosis in these patients.[11] Similar factors have been identified as being at risk for thrombosis in essential thrombocythemia patients, and it is also thought that the structural anomalies in the platelets of ET patients and leukocyte-platelet interaction increase this risk.[12] In this study, we examined the factors affecting thrombosis in PV, ET, and PMF patients. The frequency of thrombosis in PV patients has been reported to be more common in those diagnosed with ET and PMF.[13] However, no difference was found between the diagnosis and frequency of thrombosis in our study patients. In a recent study, ECLAP (European Collaboration on Low-dose Aspirin in Polycythemia Vera), it was reported that

Table 5. The relationship between JAK mutation, allele burden and the number of thrombotic events

Parameters	No thrombosis	1 thrombosis	2 thromboses	3 thromboses	p
JAK2 V617F mutation (absent/present)	32/132	6/27	4/5	0/2	0.270
JAK mutation allele burden (1-24/25-49/50-74/75-100)	50/42/18/22	6/11/5/4	3/1/0/1	2/0/0/0	0.553

arterial thrombosis was more common in male PV patients. In the same study, the frequency of splanchnic venous thrombotic events was found to be higher in women.[14] In our study, there was a statistically significant difference in favor of the male gender in terms of the frequency of thrombosis (p= 0.061). CMNs are seen more frequently with increasing age and the median age of diagnosis is 60.[15] In our study, the median age was found to be 64, similar to the general population. Advanced age is associated with a dramatic increase in the rates of venous and arterial thrombotic events. It is also believed that thrombosis frequency increases with age in myeloproliferative disorders.[5,16] However, in our study, we could not reach such a conclusion. No statistically significant difference was found between the group of patients who had thrombosis and the group who did not have thrombosis in terms of age (p= 0.800).

The relationship between thrombotic complications and JAK mutation in CMNs is unclear. The relationship between mutation allele burden and thrombosis has been investigated through PV patients, the majority of whom are JAK-positive. A relative risk of 3.56 was found for thrombosis in cases where the mutation rate was greater than 75%. In another study, patients with a mutation burden of more than 50% were examined, but no difference was found in the rate of thrombosis. A meta-analysis published in 2009 reported that thrombosis was more common in JAK mutation-positive ET patients than in negative ones.[9] Studies have also shown an increase in thrombosis frequency as the allele percentage increases.[17] Unlike a study that associates the presence of mutations with thrombosis risk in PMF, there is also a study that does not find any relationship.[7,8] In our study, we did not find a statistically significant relationship between JAK mutation and mutation allele burden and thrombosis risk, both for all patients and separately for ET and PMF patients, as a result of our analyses. The fact that the results of previous studies contradict each other and our own results indicate that more investigation is needed to prove or disprove the hypothesis that JAK mutation is associated with thrombosis. Proteolytic enzymes and reactive oxygen species that can cause damage to platelets and endothelium are produced more in situations of neutrophilia and, consequently, leukocytosis, which can result in increased formation of blood clots.[18] This is thought to be one of the reasons for the increased risk of thrombosis in CMNs. However, our study did not find a statistically significant relationship between leukocyte count and the development of thrombosis, which does not support this hypothesis. In the study, there was no significant association found between hematocrit levels at diagnosis and thrombosis.[19] High hematocrit levels are generally considered a marker of hyperviscosity and have been associated with an increased risk of thrombosis. However, in this particular study, no such relationship was found. Studies have shown that excessive increase in platelet count reduces the risk of thrombosis. This has been attributed to acquired von Willebrand disease.20 However, in our study, we did not find a statistically significant relationship between platelet count and thrombosis risk.

An increase in total protein levels contributes to hyperviscosity, and is therefore associated with a higher frequency of thrombotic events.[21] In our study, there was a borderline significant difference in total protein values between the group that experienced thrombosis and the group that did not. However, the median and mean total protein values were higher in the group that did not experience thrombosis. Infection activates the coagulation system through pathways such as platelet activation and increased tissue factor release. In a study examining the effects of infection on coagulation,

the risk of thrombotic events was found to be three times higher in cases of sepsis.[22] However, it is suggested that C-reactive protein, a biomarker that increases especially during infection, cannot predict thrombotic risk.[23] In our study, a univariate logistic regression analysis showed a relationship between sedimentation and C-reactive protein levels and thrombosis. When interpreting these results, it should be considered that there are many clinical conditions where sedimentation and C-reactive protein values can be high. Cytoreductive therapy is recommended for high-risk patient groups. The high risk is determined mainly by age and a history of previous thrombosis.[19] In our study, a borderline significant association was observed between cytoreductive therapy and thrombosis. This could be related to the fact that cytoreductive therapy is already given to the high-risk patient group.

It is known that cardiovascular risk factors such as smoking, hyperlipidemia, hypertension, and diabetes are independent risk factors for thrombosis development in patients with CMNs also.[24] Cigarette smoking primarily facilitates thrombosis through endothelial dysfunction and impaired fibrinolysis.[25] However, in our study, no association was found between smoking and thrombotic risk in Ph- CMN patients. One of the important factors in the etiology of atherosclerosis is hyperlipidemia. It also has prothrombotic effects such as decreased fibrinolysis and increased procoagulant activity.[26] When we examined the relationship between hyperlipidemia and thrombosis in Ph- CMN patients, a statistically significant relationship was found. Although the presence of hyperlipidemia is associated with an increased risk of thrombosis, no statistically significant relationship was found between lipid profile and thrombosis in our study. We believe that the lack of data on when patients started lipid-lowering therapy may be the reason for this discrepancy. It is known that diabetes, one of the cardiovascular risk factors, also causes an increased susceptibility to thrombosis due to endothelial damage. In addition, mechanisms such as activation of coagulation and platelet hyper reactivity also contribute to a prothrombotic state. Especially diabetic platelets are characterized by irregularities in various signaling pathways that lead to enhanced adhesion, activation, and aggregation. These changes arise from the interaction between hyperglycemia, insulin resistance, inflammation, and oxidative stress.[27] In this study, we found a statistically significant association between the presence of diabetes mellitus, which also accompanies Ph- CMNs, and thrombosis risk. Hypertension is an important risk factor for cardiovascular disease and also increases the risk of thrombosis in patients with CMN.[24] Hypertension is one the most common risk factors in CMN patients.[28] We also found a statistically significant relationship between hypertension and the development of thrombosis. Hypercalcemia is another parameter that needs to be studied and carefully monitored in clinical practice as it increases the risk of thrombosis. Hypercalcemia contributes to thrombosis by increasing vascular calcification, activation of coagulation factors, and platelet aggregation. Additionally, the cytotoxic and vasoconstrictive effects of hypercalcemia are believed to play a role in increasing the frequency of thrombosis, although the pathophysiological mechanisms are not yet fully understood.[29,30]

Debates continue regarding the impact of JAK mutations and allele burden on thrombosis in Ph-negative CMN patients. Previous risk stratification for thrombosis in essential thrombocythemia patients is based on the absence or presence of either age > 60 years or history of thrombosis.[31] The International Prognostic Score for Thrombosis in ET (IPSET-thrombosis), has also identified independent prothrombotic role for cardiovascular risk factors and JAK2V617F mutation.[32] The important aspect of our study is that we found no relationship between JAK mutations and thrombosis. Also our study has a few limitations.

One of the limitations of the study is its retrospective design and file scanning method. Although information on some patients having experienced thrombosis was noted, the dates of thrombosis and the start of treatment were not known. Therefore, it was not possible to evaluate whether future thrombosis could be prevented with lipid-lowering therapy. In order to avoid the results of other diseases that could cause thrombosis, the hereditary thrombophilia panel, protein C, protein S and antithrombin III levels, anticardiolipin antibody, lupus anticoagulant, antiphospholipid antibody, and anti-beta-2-glycoprotein1 levels were also planned

to be evaluated, but results could only be obtained for a few patients. Data on when patients started lipid-lowering therapy were not available. Organ sizes of patients without hepatosplenomegaly were not reported with length units, only noted as "normal". Therefore, liver and spleen size could only be evaluated in patients with hepatosplenomegaly. All laboratory parameters of all patients could not be obtained.

Conclusion

The results we obtained from our study show that there is no relationship between JAK mutations and JAK2 allele burden with the development of thrombosis in Ph-negative CMN patients. On the other hand, gender, presence of diabetes and hypercalcemia, which were found to be associated with thrombosis, may have potential for use in the thrombosis risk analysis of the Ph- CMN patients. In this study cardiovascular risk factors such as hypertension, hyperlipidemia, and diabetes were found as important risk factors for thrombosis in this patient group. The modifiability of these factors makes it even more important to inquire about accompanying illnesses. In conclusion, cardiovascular risk factors were found to be related with thrombosis in Ph- CMN rather than presence of JAK2 mutation or JAK2 allele burden. Thus, in the management of CMNs, a holistic approach including the evaluation of cardiovascular risks is needed to prevent the thrombotic events.

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