Is JAK2V617F Mutation the Only Factor for Thrombosis in Philadelphia-Negative Chronic Myeloproliferative Neoplasms?

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Received: 31 March 2015 / Accepted: 5 August 2015 / Published online: 20 August 2015
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Abstract The most common genetic disorder in Philadelphia negative chronic myeloproliferative neoplasms is the JAK2-V617F mutation. In the present study, we aimed to determine risk factors for thrombosis in patients with essential thrombocythemia and polycythemia vera. We screened the medical records of 101 patients. Risk factors which may predict thrombosis were recorded. Venous thrombosis (VT) before diagnosis was significantly higher in JAK2 positive patients. VT after diagnosis was similar in JAK2 positive and negative groups, and was significantly higher in elderly patients. Treatment places importance on the JAK2 mutation under unmodifiable cardiovascular risk factors such as advanced age after diagnosis.

Keywords Chronic myeloproliferative neoplasms · JAK2 mutation · Allele burden · Thrombosis

Introduction

Essential thrombocythemia (ET), Polycythemia vera (PV), and primary myelofibrosis (PMF) were thought to be components of the same disease when first characterized by Damashek [1]. Damashek's foresight in 1951 was proven

after the demonstration of the JAK2 mutation in chronic myeloproliferative neoplasms (CMPNs) [2]. Essential thrombocythemia (ET), and Polycythemia vera (PV) are the two most common Philadelphia negative (Ph(–)) CMPNs. Propensity to transform into acute leukemia, as well as markedly elevated thrombotic and/or hemorrhagic complications, are seen in natural disease progression [3]. Thrombotic complications remain at the heart of disease-related complications, even in the developing medication area [4]. Numerous mutations have been discovered which affect signaling pathways, epigenetic regulators, and spliceosome proteins in Ph(–) CMPNs. The most common genetic disorder in CMPNs is the JAK2 V617F mutation, while the CMPN phenotype is mainly mediated by an abnormal activation of JAK2 signal. JAK2 V617F mutations have also been demonstrated in refractory anemia with ringed sideroblasts associated with thrombocytosis, chronic myelomonocytic leukemia, acute myeloid leukemia, myelodysplastic syndrome, and chronic myeloid leukemia, respectively [5].

Phenotypic projections of JAK2 V617F mutation, including elevated leukocyte count, elderly presentation, and elevated thrombotic complications have been demonstrated in several studies [6–9]. Although thrombotic complications seen in PV are not totally explained by JAK2 V617F mutation, the JAK2 V617F mutation in ET has been found to be especially closely related with venous thrombotic complications. Also, using semi-quantitative allele determination techniques, elevated allele burden has been found to be related with thrombosis in both PV and ET [10–14]. A homozygous pattern in patients with PV, defined by >50 or >75 % of peripheral blood mononuclear cells bearing the JAK2 V617F mutation, demonstrated higher hemoglobin levels, pruritus, tendency to fibrotic transformation, and the progression of the hematologic

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disease compared to their heterozygous counterparts [15, 16].

The life expectancy of patients with ET and PV is closely related to disease-related hemostatic complications, especially thrombosis [17]. The pathogenesis of thrombosis in ET, and PV is complex and multifaceted [17]. In addition to the JAK2 V617F mutation status, many other factors may be responsible for thrombotic complications in Ph(–) CMPN. The objectives of our study were: (1) to determine risk factors for venous thrombosis in patients with Ph(–) CMPN; and (2) to figure out the role of JAK2 V617F allele burden on thrombosis site (venous and/or arterial), thrombosis timing (before and/or after diagnosis), and its relationship with treatment modalities.

Materials and Methods

We retrospectively screened the medical records of 155 patients diagnosed with Ph (–) CMPN between January 2002 and January 2014. CMPNs except ET, and PV and JAK2 status that could not be displayed were excluded from the study. There were 9 primary myelofibrosis (PMF) patients observed in this time period, so PMF patients were excluded due to the limited number of PMF patients to perform statistical analysis. During this period, 101 patients who were screened for JAK2 mutation were diagnosed with ET and PV. The diagnosis were based on World Health Organization 2008 criteria [18]. A bone marrow biopsy was performed on all patients. Patients' demographic features, clinical data, and JAK2 V617F mutation status were recorded. JAK2 allele burden was also evaluated. Medical records were retrospectively assessed, especially for venous/arterial thrombosis before and after treatment, looking for cigarette smoking, diabetes mellitus (DM), hypertension (HT), cytoreductive therapy, and anti-platelet treatment. Presence of thrombosis was assessed using patient-based self-reports and hospital records. We routinely screen patients who experienced thrombosis before 50 years of age for hereditary thrombophilia in our facility. Screening tests include plasma levels of Protein C/S and antithrombin III; genetic evaluation for factor V Leiden, and Prothrombin 20210 mutation; and thoracoabdominal tomography. Patients with accompanying hereditary thrombosis were excluded from the study.

JAK2 V617F mutation was investigated from the peripheral blood mononuclear cells. After obtaining DNA from granulocytes isolated from peripheral-blood samples using conventional gradient centrifugation techniques, a semi-quantitative Ipsogen JAK2 MutaScreen RS kit was used to identify JAK2 V617F/G1849T mutation. Procedures for JAK2 analysis was performed according to

manufacturer instructions. The study was conducted in accordance with the Declaration of Helsinki Ethical Principles for Medical Research. Patients gave informed consent. Approval was obtained from the Trakya University ethical committee.

Statistical analysis was performed using SPSS version 17. Kolmogorov–Smirnov test was used for normality assessment. Differences between categorical parameters were calculated by Chi square test. Event-free survival was calculated using Kaplan–Meier survival analysis.

Results

Socio-Demographic Findings

Socio-demographic findings are summarized in Table 1. Of 101 patients, 76.3 % ($n = 74$) had ET, while 26.7 % ($n = 27$) had PV. Mean age at diagnosis was 61.7 ± 12.8 (28–87) years, and the mean disease duration was 56.1 ± 33.8 (12–180) months. At the time of initial diagnosis, 10.9 % ($n = 11$) patients had DM, while 46.5 % ($n = 47$) patients had HT. The cigarette smoking rate was 30.7 % ($n = 31$). 76.3 % ($n = 74$) of the patients were JAK2 V617F positive.

All Patients

Mean time from thrombosis to diagnosis was 43.8 ± 41.0 months, and duration from diagnosis to thrombosis was 22.3 ± 21.2 months in patients with preceding thrombosis after diagnosis. Thrombotic attacks were observed in 62.4 % of the patients ($n = 63$). Thrombosis was observed in 5.9 % of the patients ($n = 6$) both before and after diagnosis, while 21.8 % ($n = 22$) had thrombosis solely before, and 9.9 % ($n = 10$) had thrombosis solely after diagnosis. Smoking did not impose a higher risk of thrombosis ($p = 0.716$). Hypertensive patients experienced more frequent thrombosis than normotensives ($p = 0.041$). The frequencies of thrombosis in hypertensive and normotensive CMPN patients after diagnosis were similar. DM did not turn out to be a risk factor for thrombosis.

There was no difference in total thrombosis before and after diagnosis in patients younger (31.1 % ($n = 31/45$)) or those older than 60 years (42.9 % ($n = 24/56$)). There was no difference in thrombosis frequency before diagnosis between patients younger or older than 60 years. Thrombosis after diagnosis was significantly higher in patients over 60 years old (14/56, 25 %) than younger counterparts (2/45, 4.4 %) ($p = 0.006$). Thrombosis frequency did not show any difference in patients with leukocytosis.

Table 1 Socio-demographic findings of Ph negative chronic myelo-proliferative neoplasms

	All CMPN	ET	PV	p
Age	61.7 ± 12.8	60.2 ± 13.4	60.9 ± 13.5	0.32
Disease duration (m)	56.1 ± 33.8	58.2 ± 35.4	50.4 ± 28.7	0.26
Smoking duration (m)	87.6 ± 139.7	71.8 ± 128.4	135.0 ± 163.3	0.09
HT duration (m)	52.7 ± 69.5	47.3 ± 69.5	67.1 ± 68.7	0.21
DM duration (m)	94.8 ± 40.5	79.2 ± 28.9	110.4 ± 47.5	0.25
SS (cm with USG)	13.4 ± 2.4	13.2 ± 2.2	13.3 ± 2.5	0.95
TBD. (m)	43.8 ± 41.0	44.2 ± 43.3	43.2 ± 38.7	0.95
TAD. (m)	22.3 ± 21.2	20.5 ± 18.6	26.0 ± 27.7	0.65

CMPN Chronic myeloproliferative neoplasms, *ET* essential thrombocythosis, *PV* polycythemia vera, *PMF* Primary myelofibrosis, *HT* hypertension, *DM* diabetes mellitus, *SS* spleen size, *TBD* thrombosis before diagnosis, *TAD* thrombosis after diagnosis, *cm* centimeter, *m* months

The relationship between thrombosis timing and JAK2 status is shown at Table 2. Patients with JAK2 V617F mutation had a significantly higher total thrombotic attack than those without ($p < 0.005$). Thrombotic attacks before diagnosis was significantly higher in JAK2 V617F positive patients than wild type carriers ($p = 0.009$). Table 3 summarizes the relationship between JAK2 V617F allele burden and specialties of thrombosis such as timing and the thrombosis site. Venous thrombosis before the diagnosis showed no difference between JAK2 V617F negative and positive groups, while arterial thrombosis was significantly higher in JAK2 V617F positive group ($p = 0.03$). Incidences of thrombosis after diagnosis were similar between JAK2 V617F positive and negative groups ($p = 0.06$). Venous thrombosis after the diagnosis was similar between groups, ($p = 0.08$), as was arterial thrombosis ($p = 0.057$).

Arterial thrombosis was significantly higher in patients with 12.6–30 %, and 78–100 % JAK2 V617F allele burden ($p = 0.001$). For both these allele burdens, all thrombotic events were arterial events. Thrombotic events both before and after diagnosis showed no difference between homozygous (>50 % JAK2 V617F bearing peripheral mono-nuclear cells) and heterozygous patients.

We retrospectively examined D-dimer levels at admission to evaluate its predictive value in thrombosis. Mean D-dimer value at admission in patients who did not experienced thrombosis after diagnosis was $1.05 \pm 1.34 \mu\text{g/mL}$. D-dimer value at admission in patients who did experienced thrombosis after diagnosis was $1.85 \pm 1.40 \mu\text{g/mL}$. D-dimer value at admission was

significantly higher in patients who experienced thrombosis after diagnosis ($p = 0.013$).

Event free survival was calculated in patients with both ET and PV; there was no difference in event free survival between patients under or above 60 years of age, JAK2 V617F positive or negative groups, JAK2 allele burden (homozygous or heterozygous), whether patients experienced thrombosis before diagnosis or not, leukocyte count elevation, and use of cytoreductive therapy.

Treatment

Phlebotomy was used in 82.8 % ($n = 24/29$) of PV patients. Cytoreductive therapy was used in 88.1 % ($n = 89$) of the patients to control the disease. Cytoreductive therapy was used in 91.9 % ($n = 68$) of ET, and 77.8 % ($n = 21$) of PV patients. Thrombosis was observed in 16.7 % (2/12) of the patients not using cytoreductive therapy, and in 40.4 % (36/89) of the patients who were using cytoreductive therapy. There was no difference in thrombosis in patients in terms of cytoreductive therapy status ($p = 0.20$). There was also no difference in occurrence of thrombotic events before diagnosis in both groups.

Sub-Group Analysis

When ET patients were examined as a separate group, there was no difference in classical cardiovascular risk factors, whether they experienced thrombosis or not. Thrombosis free from timing did not result in any differences between patients either above or below 60 years of age in ET ($p = 0.092$). JAK2 V617F positivity was observed in 75.6 % ($n = 31$) of the ET patients over 65 of age. 19.5 % ($n = 8$) of the ET patients have homozygous JAK2 allele burden. JAK2 allele positivity ($p = 0.028$) and homozygous allele burden ($p = 0.037$) were higher in elder ET than younger patients. Elevated leukocyte count at diagnosis was significantly higher in patients with JAK2

Table 2 Relationship between Thrombosis and JAK2 Positivity

	TT	TBD	TAD
JAK2 negative	4 (14.8 %)	2 (7.4 %)	2 (7.4 %)
JAK2 positive	34 (45.9 %)	25 (33.8 %)	14 (18.9 %)

TT Total thrombosis (=number of patients experienced thrombosis), *TBD* thrombosis before diagnosis, *TAD* thrombosis after diagnosis

Table 3 Relationship between thrombosis and JAK2 allele burden

(%)	TT	TBD	TAD
0	4 (14.8 %)	2 (7.4 %) [7.4 % A]	2 (7.4 %) [3.7 % A/3.7 % V]
0–12.5	7 (63.6 %)	5 (45.5 %) [27.3 % A/18.2 % V]	4 (36.4 %) [18.2 % A/18.2 % V]
12.6–30	6 (42.9 %)	4 (28.6 %) [28.6 % A]	3 (21.4 %) [21.4 % A]
31–50	13 (41.9 %)	13 (41.9 %) [38.7 % A/3.2 % V]	2 (6.5 %) [3.2 % A/3.2 % V]
51–77	4 (50 %)	2 (18.2 %) [9.1 % A/9.1 % V]	3 (27.3 %) [9.1 % A/18.2 % V]
78–100	3 (42.9 %)	1 (14.3 %) [14.3 % A]	2 (28.6 %) [28.6 % A]

Bold values indicate statistically significant $p > 0.05$

A Arterial type thrombosis, TT total thrombosis (=Number of patients experienced thrombosis), TBD thrombosis before diagnosis, TAD thrombosis after diagnosis, V venous type thrombosis

V617F positive patients than wild type carriers ($p = 0.01$). JAK2 V617F positive ET patients demonstrated higher total thrombotic events than negative patients ($p = 0.006$). Thrombotic events before diagnosis in JAK2 V617F positive patients ($n = 16/47$) with ET was significantly higher than negative patients ($n = 2/27$) ($p = 0.01$). Thrombosis after diagnosis was similar between JAK2 V617F positive and negative patients. High risk patients demonstrated higher JAK2 V617F positivity than low risk patients ($p = 0.018$). Thrombotic complications did not differ whether the JAK2 V617F allele burden was homozygous or heterozygous. Classical cardiovascular risk factors were evaluated in both low risk and high risk patients.

When PV patients are taken as a separate group, thrombotic events regardless of timing have been shown to be similar in patients above 60 years of age than younger patients ($p = 0.707$). Thrombotic events before or after diagnosis were similar between age groups. Homozygous and heterozygous patients due to JAK2 V617F allele burden did not show any difference in thrombotic events in patients with PV.

Discussion

First of all, our study has demonstrated elevated levels of arterial thrombosis in JAK2 V617F positive patients than negative ones. Although arterial thrombosis was significantly elevated in JAK2 V617F positive group, venous thrombosis did not differ between JAK2 V617F positive and negative groups. The presence of the JAK2 V617F mutation has been shown to affect disease phenotype. For example, association between elevated leukocyte count, elderly presentation, and elevated thrombotic complications with JAK2 V617F positivity has been demonstrated in patients with CMPNs [6–9]. Recently published three meta-analysis evaluated the tendency to thrombosis in JAK2 V617F positive ET patients [10–12]. In a meta-analysis, Lussana F et al. demonstrated a twice-elevated risk of either arterial or venous thrombosis in patients with a JAK2 V617F mutation with ET [12]. Although many

studies have demonstrated the arterial and venous thrombosis tendency in JAK2 V617F positive ET, thrombosis tendency in JAK2 V617F positive PV is still an area of controversy. On the other hand, a study by Passamonti F et al. found out no association of thrombosis with JAK2 V617F mutation in PV [19].

The present study demonstrated that the presence of JAK2 V617F mutation elevated the risk of thrombosis before diagnosis, whereas JAK2V617F mutation did not elevate the risk of thrombosis after diagnosis. Patients over 60 years of age experienced more thrombosis after diagnosis than other groups. We think that this finding may be due to the effects of antithrombotic and cytoreductive therapy that puts the importance of JAK2V617F mutation under the unmodifiable cardiovascular risk factor, advanced age, after diagnosis. Low dose aspirin has been demonstrated decreasing both arterial and venous thrombosis in ET and PV [20, 21]. Hydroxyurea has been shown to reduce the rate of thrombosis, especially in cerebral transient attacks in ET [22]. On the other hand, the efficacies of IFN- α and anagrelide were not well known. Anagrelide was studied in two randomized trials. Although PT-1 trial demonstrated elevated arterial thrombosis with anagrelide [23], ANAHDRET study demonstrated equal arterial and venous thrombosis frequency with hydroxyurea [24]. Although in our study patients using cytoreductive therapy did not experience more thrombosis than their opponents, it seems like this that therapy itself could change the risk ranking to predict thrombosis. The present study has demonstrated that modifiable classical cardiovascular risk factors like smoking, DM, dyslipidemia, and HT play limited roles for prediction of thrombotic attacks. Classical atherosclerotic risk factors have been evaluated in patients with CMPNs. These risk factors were demonstrated to put low risk patients to intermediate or high risk group by a group of authors [23, 25]. In our study, none of these modifiable classical risk factors were seen to elevate the thrombosis risk in CMPNs patients.

One of the indicator of coagulation activation is D-dimer, a specific cross-linked fibrin product. Although highly sensitive to thrombosis, its specificity is lower,

meaning that false positive results may be observed in wide variety of inflammatory and prothrombotic conditions [26]. In our study, mean D-dimer value at admission of patients who would be experience thrombosis after diagnosis is higher than patients who would not experience thrombosis after diagnosis. Our results should be interpreted carefully, because D-dimer results may demonstrate difference even within the same laboratory mainly due to instrumentation and calibration differences. It was not possible to rule out these unfavorable factors due to the retrospective nature of our study.

In our study we also evaluated the impact of JAK2 V617F allele burden on thrombosis site, and found out that arterial thrombosis not venous one is elevated with allele burden between 12.6–30 % and 76–100 %. Our study was incapable to demonstrate elevated thrombosis risk with homozygous patients than their opponents. Vannucchi et al. was able to demonstrate elevated thrombotic risk in PV patients with >75 % allele burden of JAK2 V617F mutation. [13] In a retrospective study, elevated vascular complications was shown in >50 % allele burden. [14] The widely used technique to evaluate allele burden in CMPNs is determination of the presence of JAK2 V617F mutation on peripheral mononuclear cells, but homozygosity can be only demonstrated by evaluating the presence of clonality within JAK2 V617F positive cells. For example, in a large population of granulocytes, a homozygous clone may be falsely determined to be heterozygous. Contrarily, a heterozygous clone may be determined to be falsely homozygous in presence of granulocytopenia. Due to the rationale above, a cut of value such as 50 or 75 % may cause false positive or false negative results.

The limitations of our study are (1) retrospective design; (2) small sample size, especially in the PV group; (3) impossibility of treatment randomization; and (4) inability to evaluate clonality from clonal genotyping. In our study, the number of PV patients was approximately half that of ET patients. The reason for this unexpected result was that we could not screen patients diagnosed before 2008 for JAK2 V617F mutation. Because JAK2 V617F mutation is essential for PV but not ET, the number of patients diagnosed with ET before 2008 was significantly higher than that of patients with PV. Prospective studies with treatment randomization should be performed to evaluate the missing ring, demonstrating the impact of JAK2 V617F mutation and its allele burden on CMPNs.

In conclusion, it has been demonstrated that JAK2V617F mutation elevates thrombosis risk, especially before diagnosis. Modifiable risk factors may have a low impact on thrombogenic risk. Elderly patients showed elevated risk of thrombosis after diagnosis. JAK2 V617F allele burden had no impact on thrombosis timing. Allele burden could predict the site of thrombosis, whether

arterial or venous. This phenomenon should be evaluated in larger prospective trials.

Acknowledgments This work was supported by authors themselves.

Authors Contributions MS.U., M.B. and G.E.P. designed the study; M.B., M.M. and E.G.U. provided the patients' samples; M.S.U. and M.A. performed statistical analysis, M.A. designed the tables; A.M.D. and E.A. performed the experiments and analyzed the data; E.A. and G.E.P. reviewed the manuscript. All authors contributed to the writing of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interests.

Ethical Standards The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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