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Association between Thromboembolic Events and the *JAK2* V617F Mutation in Myeloproliferative Neoplasms

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Summary: Thrombotic complications are a major cause of death in patients with Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs), which are closely associated with the *JAK2* V617F activating mutation. However, whether the presence of the *JAK2* V617F mutation affects thrombotic risk is currently unknown, although some reports have suggested a variable association with thrombosis. Therefore, we investigated the association between *JAK2* V617F and various complications, including thrombosis, in Japanese patients with MPNs. We assessed the *JAK2* V617F status in 140 patients who were diagnosed or doubted as having some type of MPN by utilizing a *JAK2* V617F-specific guanine-quenching probe. *JAK2* V617F was detected in 31 of 51 patients (60.8%) with essential thrombocythemia, all 16 patients (100%) with polycythemia vera, 4 of 11 patients (36.4%) with primary myelofibrosis, 2 of 18 patients (11.1%) with other types of MPNs, and none of the 44 patients with doubted MPN. In the 78 patients with classical MPN, *JAK2* V617F correlated with a leukocyte count ≥10,000/μl (p=0.046). Complications of thrombosis, hemorrhage, and leukemic transformation occurred in 21 (41.2%), 4 (25.0%), and 3 (27.3%) patients with classical MPN, respectively, and thrombotic events (TE) occurred more frequently in patients with *JAK2* V617F than without (p=0.047). Based on these findings, initial screening for the *JAK2* mutation and careful monitoring for thrombotic events should be performed in patients with MPN.

Key words polycythemia vera, essential thrombocythemia, primary myelofibrosis, thrombosis, somatic mutation

INTRODUCTION

Philadelphia chromosome-negative MPNs are hematological malignancies characterized by extensive proliferation of multipotent myeloid progenitor cells and include chronic myelomonocytic leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The molecular basis of MPNs, excluding CML, was largely elusive until 2005, when several research groups reported a novel acquired somatic mutation of *JAK2* gene [1-3]. A single G to T somatic point mutation at nucleotide 1,849 in exon 14 of *JAK2* results in the substitution of valine with phenylalanine at codon 617 (*JAK2* V617F). The mutation is present in the JH2 autoinhibitory domain of the *JAK2* tyrosine kinase protein.

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Abbreviations: CEL, chronic eosinophilic leukemia; CML, chronic myelogenous leukemia; CMMoL, Chronic myelomonocytic leukemia; CNL, chronic neutrophilic leukemia; ET, essential thrombocythemia; *JAK2*, Janus kinase 2 gene; MDS, myelodysplastic syndrome. *MPL*, thrombopoietin receptor gene; MPNs, myeloproliferative neoplasms; PMF, primary myelofibrosis; PV, polycythemia vera; *TET2*, ten-eleven translocation 2 gene.

The *JAK2* V617F results in a gain-of-function of *JAK2* which, growth factor-independently, activates downstream signaling pathways including JAK-STAT, PI3K/Akt, and ERK1/2 MAPK, leading to abnormal expansion of myeloid cells. *JAK2* V617F has been identified in nearly all PV patients and in approximately half of all ET and PMF patients [4-7]. Multiple mouse models that recapitulate the human MPN phenotype on expression of the *JAK2* V617F allele have demonstrated that *JAK2* V617F is central to the pathogenesis of MPNs. Other acquired mutations have since been found to be involved in the pathogenesis of MPNs, such as mutations and deletions in exon 12 of *JAK2* [8] and mutations in the *MPL* [9] or TET2 gene [10]. The role of the other low-frequency mutations in the pathogenesis of MPNs and their interplay with the *JAK2* mutation are unclear. It is now well known that MPNs share a common stem cell-derived clonal heritage and that their phenotypic diversity is attributed to different configurations of abnormal signal transduction, resulting from a spectrum of mutations affecting protein tyrosine kinases or related molecules [11,12]. Therefore, the classification and criteria for diagnosing MPNs were updated by the World Health Organization (WHO) in 2008 [13].

The three common or classic MPNs-PV, ET, and PMF-share several clinical characteristics such as chronic proliferation of myeloid cells in bone marrow and peripheral blood and a propensity for thrombosis and hemorrhage. However, some patients exhibit clinical signs of disease progression, demonstrating the variable stage of secondary myelofibrosis and pancytopenia. Moreover, transformation to acute leukemia is observed in approximately 10% of cases.

Many studies have been performed to evaluate the association of *JAK2* V617F with the risk of thrombosis, especially in patients with ET, but the results have been inconclusive [14-17].

Considering that thrombosis is a major cause of morbidity and mortality in patients with MPNs, the identification of a biological marker for high-risk patients with MPNs would be of great clinical utility.

Therefore, we investigated the incidence and characteristics of complications, including thrombosis, in patients with MPNs and studied the association of *JAK2* V617F with thrombosis.

METHODS

Patients

The presence of *JAK2* V617F in DNA from peripheral blood leukocytes was retrospectively investigated in 140 patients with erythrocytosis, leukocytosis, and/or thrombocytosis after obtaining informed

TABLE 1.
Clinical features of 140 patients screened for the Janus kinase 2 (JAK2) V617F mutation

Diagnosis	*n*	*Frequency of JAK2 V617F mutation*	*Frequency of thrombosis (with or without the JAK2 V617F mutation)*	*p value*
Total	140	37.9%	30.2% / 9.2%	0.001
Classical MPN	78	65.4%	31.4% / 11.1%	0.047
PV	16	100%	8.6% / –	–
ET	51	60.8%	41.9% / 10.0%	0.015
PMF	11	36.4%	0% / 14.3%	0.428
Other MPN/MDS	18	11.1%	0% / 12.5%	0.716
MDS	10	10.0%	0% / 0%	–
CMMoL	1	0%	– / 0%	–
CML	4	0%	0% / 33.3%	–
CNL	1	100%	0% / –	–
CEL	2	0%	_ / 0%	_
Secondary erythrocytosis, thrombocythemia, or leukocytosis	44	0%	_ / 9.1%	_

Abbreviations: MPNs, myeloproliferative neoplasms; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; MDS, myelodysplastic syndrome; CMMoL, chronic myelomonocytic leukemia; CML, chronic myelogenous leukemia; CNL, chronic neutrophilic leukemia; CEL, chronic eosinophilic leukemia.

consent. The patients were recruited from our hospital and affiliated hospitals from April 2010 to December 2012. Mean age was 60.4 years (range, 12-86 years), and the male/female ratio was 63.6%. All patients were classified according to the 2008 WHO criteria for MPNs [18]. The included patients were diagnosed as having PV (n=16), ET (n=51), PMF (n=11), myelodysplastic syndromes (MDS, n=10), chronic myelomonocytic leukemia (CMMoL, n=1), CML (n=4), chronic neutrophilic leukemia (**CNL, n=1**), or chronic eosinophilic leukemia (**CEL, n=**2) (Table 1). The other 44 patients were categorized as having a reactive and secondary increase in blood cells.

The characteristics of the 78 MPN patients (with PV, ET, and/or PMF) are shown in Table 2. Therapeutic drugs, including cytoreductive and thromboprophylactic agents, were administered to most patients with thrombosis (Table 3).

Complications such as thrombosis, hemorrhage, and progression to leukemia were reviewed at a mean follow up of 65 months (range, 1-293 months). Thrombosis included arterial or venous thrombosis and ischemic events, such as acute myocardial infarction, ischemic stroke, cerebral transient ischemic attack, peripheral arterial thrombosis, and venous thrombosis. Hemorrhage included purpura, intracranial bleed-

TABLE 2.
Laboratory findings at diagnosis and complications

Characteristics	Total n=78		Pvalue	PV n=16	ET n=51		PMF n=11	
	*JAK2*V617F n=51	Wild type n=27	* x^2-test **t-test	*JAK2*V617F n=16	*JAK2*V617F n=31	Wild type n=20	*JAK2*V617F n=4	Wild type n=7
Male / Female	26 /25	10 /15	0.368*	5 /11	18 /13	9 /11	3 /1	3 /4
Age (years) Mean (range)	67.0 (20-86)	55.4 (12-80)	0.003**	63.6 (20-81)	68.8 (28-86)	50.6 (12-80)	66.5 (62-73)	69.3 (51-79)
WBC (/μl) Mean	13,525	9,391	0.131**	13,018	14,448	10,345	8,400	6,666
<10,000/μl (n)	20	17		6	11	12	3	5
≧10,000/μl (n)	31	10		10	20	8	1	2
			0.046*					
Hemoglobin (g/dl) Mean	15.0	11.9	0.190**	17.1	14.5	12.6	10.6	10.0
Platlets ($\times10^3$/μl) Mean	721	648	0.789**	514	895	798	194	219
<450×10^3/μ (n)	12	8		8	0	1	4	7
≧450×10^3/μ (n)	39	19		8	31	19	0	0
			0.557*					
LDH (U/l) Mean	336	338	0.636**	329	312	314	557	329
<230U/l(n)	13	6		3	8	6	2	0
≧230U/l(n)	38	17		13	23	10	2	7
			0.019*					
Complications (n)	20	8	0.401*	4	14	7	2	1
Thrombosis (n)	16	3	0.047*	3	13	2	0	1
Cerebral infarction/ another arterial thrombosis/venous thrombosis (n)	9 / 4 / 3	1 / 1 / 1	0.053 (A)* 0.678 (V)*	3 / 0 / 0	6 / 3 / 4	1 / 0 / 2	0 / 0 / 0	0 / 0 / 1
Hemorrhage (n)	4	4	0.334*	2	2	4	0	0
Progression to leukemia (n)	5	2	0.725*	0	3	2	2	0

TABLE 3.
Characteristics of myeloproliferative neoplasm patients with thrombosis

Patient No.	Duration of diagnosis and to the day of the *JAK2*V617F test (months)	Follow up (months)	*JAK2* V617F	*JAK2*V617F allele burden (%)	Diagnosis	Age (Diagnosed)	Age (tested *JAK2* status)	Sex	Blood cell count			Treatment		Hypertension	Dyslipidemia	Diabetes mellitus	Thrombotic complication
									WBC (/μl)	Hb (g/dl)	Plt ($\times 10^3$/μl)	HU	Anticoagulation				
1	0	16	(–)		ET	80	80	M	10,200	13.8	997	(–)	Aspirin	(+)	(+)	(+)	CI
2	0	23	(–)		ET	59	59	M	15,100	16.4	456	(–)	Aspirin	(+)	(–)	(+)	ASO
3	2	13	(–)		PMF	72	72	M	16,100	8.7	43	(+)	(–)	(+)	(+)	(+)	Splanchnic vein thrombosis
4	118	127	(+)	76.6	ET	72	82	F	10,900	13.9	1,201	(+)	Aspirin	(–)	(–)	(+)	CI
5	6	27	(+)	74.8	ET	76	76	F	21,300	15.6	658	(+)	Aspirin	(+)	(+)	(–)	CI
6	0	19	(+)	76.2	ET	80	80	F	22,300	15.8	977	(+)	Clopidogrel	(+)	(–)	(–)	CI
7	0	11	(+)	78.5	ET	83	83	F	27,800	15	108	(+)	(–)	(–)	(–)	(–)	CI
8	28	52	(+)	23.8	ET	71	73	F	9,080	15.4	863	(+)	Aspirin	(–)	(–)	(–)	Lower extremity DVT
9	0	1	(+)	23.6	ET	79	79	F	11,700	15.1	1,297	(+)	Aspirin	(–)	(–)	(+)	MI
10	25	46	(+)	16.9	ET	74	76	M	5,800	11.9	546	(+)	Clopidogrel, Warfarin		(+)	(–)	ASO, AAA
11	0	3	(+)	19.3	ET	64	64	M	9,200	18.1	1,027	(+)	Warfarin	(+)	(–)	(–)	ASO
12	163	293	(+)	11.3	ET	57	71	M	11,900	13.7	1,172	(+)	Aspirin	(–)	(–)	(–)	MI
13	127	28	(+)	10.7	ET	67	73	M	12,800	11.1	1,755	(+)	Aspirin	(+)	(+)	(+)	CI
14	80	94	(+)	40.6	ET	76	83	M	9,800	15.8	757	(+)	Aspirin	(–)	(–)	(–)	CI
15	0	1	(+)	20.6	ET	74	74	M	10,910	15.8	1,035	(+)	Aspirin	(+)	(+)	(+)	Lower extremity DVT
16	98	118	(+)	53.2	ET	75	83	M	27,600	16.6	1,294	(+)	Aspirin	(+)	(–)	(–)	Ischemic colitis
17	0	15	(+)	27	PV	77	77	F	11,400	18.2	969	(+)	Aspirin	(+)	(+)	(+)	CI
18	0	1	(+)	82	PV	54	54	M	20,100	20.4	625	(+)	Aspirin	(+)	(–)	(–)	CI
19	74	84	(+)	69.9	PV	72	78	M	8,330	13.1	248	(+)	(–)	(+)	(–)	(–)	CI

ing, gastrointestinal bleeding, and bleeding from other sites.

Analysis of the JAK2 V617F mutation

The *JAK2* V617F mutation was detected with a system utilizing a *JAK2* V617F-specific guanine-quenching probe (QP-system), a fully automated single nucleotide polymorphism genotyping system, and a prototype of i-dency™ (ARKRAY, Inc., Kyoto, Japan). The quenching probe (QProbe) is a fluorescent probe containing a fluorescent substance bound to the terminal cytosine that becomes quenched on hybridization with a complementary strand. With increasing temperature, the duplex unravels at a temperature related to the strength of the bond between the QProbe and the complementary chain, and at this temperature point, the fluorescence intensity recovers. This system utilizes a technique to determine the degree of complementation between the QProbe and the target nucleic acid by measuring the change in fluorescence intensity with increasing temperature. Here we detected the *JAK2* V617F mutation by using i-dency™. The forward and reverse polymerase chain reaction (PCR) primers flanking *JAK2* G1849 were 5'-GCAGCAAGTATGATGAGCAAGCTTTCTC-3' and 5'-GCTCTGAGAAAGGCATTAGAAAGCCTG-3', respectively. The tetramethylrhodamine-conjugated-specific guanine-quenching fluorophore probe (QProbe), which is complementary to mutant *JAK2*, was 5'-AGTATGTTTCTGTGGAGAC-(tetramethylrhodamine)-3'. To detect *JAK2* V617F, we prepared anticoagulated peripheral blood from the patients, and samples were tested using the QP-system as described previously [19]. All procedures were completed automatically.

Sensitivity of JAK2 V617F detection

The sensitivity of the QP-system for identifying low concentrations of the *JAK2* V617F allele was evaluated by mixing cell lines. Megakaryoblastic cell line Dami, which has homozygous *JAK2* V617F alleles, and the T-cell lymphoma Jurkat cell line which has homozygous *JAK2* wild-type alleles, were used. After measuring the cell count, cells were mixed in various ratios, and the DNA mixtures were analyzed using the QP-system, allele-specific PCR, and direct sequencing.

Allele-specific polymerase chain reaction and direct sequencing

Allele-specific PCR was performed by the methods described by Baxter et al. [2].

The PCR products from DNA of the cell line mixture were purified and directly sequenced on both strands using a BigDye Terminator V3.1 Cycle Sequencing Kit and an ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's instructions and using conditions previously described [20], and the sequenced products were analyzed using the ABI 310 DNA analyzer.

Statistical analysis

We compared clinical characteristics according to the status of *JAK2* using Student's *t*-test and the Chi-squared test. Student's *t*-test was used to detect significant differences between blood counts including hemoglobin, leukocytes, and platelets. For categorical variables, the correlations between the *JAK2* mutation and complications, including thrombosis, were analyzed using the Chi-squared test. Differences were considered significant at $p<0.05$. All calculations were performed using the Statistical Software Package for the Social Sciences (SPSS version 20 for Windows; SPSS, Chicago, IL).

RESULTS

Sensitivity of the QP-system for JAK2 V617F detection

We tested the *JAK2* status in 140 patients by using an automated device (i-densy™) with a *JAK2* V617F-specific guanine-quenching probe (QP-system). Dami cells and Jurkat cells were mixed at the designated ratios, and DNA was extracted from the cell mixtures. The detection sensitivity of the *JAK2* V617F mutation was assessed by three techniques-the automated QP-system, direct sequencing, and allele-specific PCR. As shown in Fig. 1, 5% of the targeted *JAK2* allele burden was detected by the QP-system and allele-specific PCR technique. Moreover, a 1% allele burden was detected by the QP-system alone, even though it was unclear in the allele-specific PCR method.

Association between clinical features and the JAK2 V617F mutation

The clinical features of the 140 patients are shown in Table 1. The *JAK2* V617F mutation was identified in 53 of the 140 patients (37.9%) who underwent screening for the *JAK2* V617F mutation. The incidence of the *JAK2* V617F mutation in each disease was as follows: 100% (16/16) in PV; 60.8% (31/51) in ET; 36.4% (4/11) in PMF; 10.0% (1/10) in MDS, and 100% (1/1) in CNL. The mutation was not detected in any of the other diseases (CML and CNL) or in any of the 44 patients with secondary erythrocytosis, thrombocythemia, and/or leukocytosis (Table 1), demonstrating the high specifi-

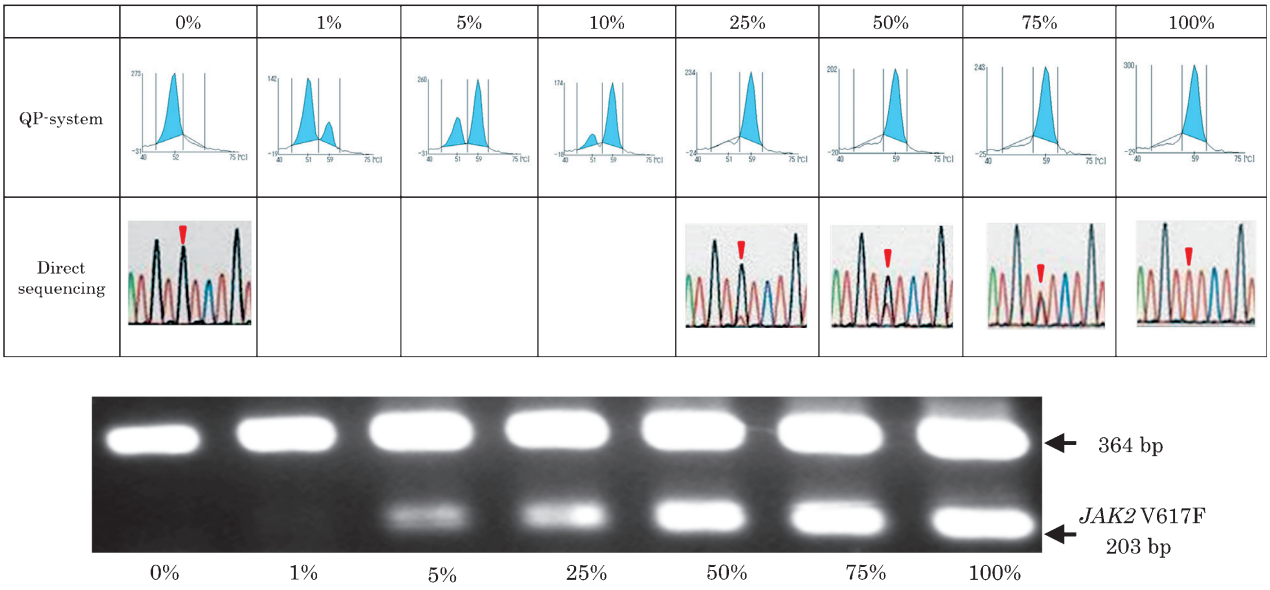

Fig. 1. Detection and sensitivity of the *JAK2* V617F mutation
Dami cells and Jurkat cells were mixed in the designated ratios, and *JAK2* V617F was analyzed by three methods. The upper panel shows the results from the QP-system. A 1% allele burden was detected by the QP-system. The middle panel shows the direct DNA sequence patterns with arrows indicating the mutation site. The lower panel shows the allele-specific polymerase chain reaction (PCR) and agarose gel electrophoresis pattern of the PCR products. The lower bands indicate the presence of the *JAK2* V617F mutation. The upper bands show the PCR products of the internal control.

city of the *JAK2* V617F mutation in classical MPNs.

Thrombotic events were observed in 30.2% of patients with *JAK2* V617F, compared with 9.2% of patients without *JAK2* V617F. The frequency of thrombotic events was significantly higher in patients with *JAK2* V617F than in those without (p=0.001). Therefore, the presence of the *JAK2* V617F mutation is considered a possible risk factor for thrombosis.

Relationships between the JAK2 V617F mutation and laboratory findings in patients with MPNs

The initial clinical and laboratory findings according to *JAK2* V617F status in the 78 patients diagnosed as having classical MPNs (PV, ET, and/or PMF) are shown in Table 2. The mean age of patients with *JAK2* V617F and without (wild type) was 67.0 (range, 20-86) and 55.4 (range, 12-80) years, respectively. The difference in age was significant (p=0.003), but not for sex distribution.

The median white blood cell (WBC) count in patients with *JAK2* V617F was 13,525/μl (range, 433-44,500/μl) and was notably higher than that in cases without *JAK2* V617F, which had a median WBC count of 9,391/μl (range, 1,920-31,900/μl), although it was not significant (p=0.131). Moreover, hemoglobin level (p=0.190) and platelet count (p=0.789) showed no significant differences between patients with and without the *JAK2* V617F mutation.

To further investigate the relationships between *JAK2* V617F and blood cell counts, patients were grouped according to WBC classification (<10,000/μl, ≥10,000/μl), platelet counts (<450×10^3/μl, ≥450×10^3μl), and lactate dehydrogenase levels(LDH) (<230 U/l, ≥230 U/l), based on the reference ranges and diagnostic criteria of the MPNs. Considering WBC count, patients were classified as follows: 20 (*JAK2* V617F, <10,000/μl), 17 (wild type, <10,000/μl), 31 (*JAK2* V617F, ≥10,000μl), and 10 (wild type, ≥10,000μl). The patients with the *JAK2* mutation had significantly higher WBC counts (≥10,000/μl) (p=0.046), as assessed by the Chi-squared test. Similarly, LDH was also significantly higher (p=0.019).

Relationships between the JAK2 V617F mutation and complications in MPN patients

Thrombotic complications, including ischemic stroke, cerebral transient ischemic attack, acute myocardial infarction, peripheral arterial thrombosis, and venous thrombosis were found in 19 patients, before or after the diagnosis (Table 2). Eight patients had bleed-

ing complications, and 7 patients progressed to acute leukemia. Of the 19 patients with thrombotic events, 16 had the *JAK2* V617F mutation, which was significantly more prevalent than in those without *JAK2* V617F (p=0.047). This was particularly evident in ET (p=0.015) (Table 1). The clinical characteristics of these patients are shown in Table 3. No significant differences were noted between the other complications and *JAK2* mutation status (Table 2).

DISCUSSION

In the present study, we investigated the relationship between the *JAK2* V617F mutation and complications in MPN patients. *JAK2* V617F status was detected using i-densy™ and the QP-system, which constitute a fully automated and super-rapid system. The QP-system detected the presence of at least a 1% mutated allele, and its sensitivity was superior or similar to that of other methods such as direct sequencing and allele-specific PCR methods (Fig. 1). Therefore, the QP-system showed sufficient sensitivity and convenience.

Screening of *JAK2* V617F was performed in 140 patients with increased blood cell counts and identified in 78 with MPNs according to the WHO diagnostic criteria [13,18]. The incidence of the mutation in PV (100%), ET (60.8%) , and PMF (36.4%) was similar to previous observations [3].

Reports on the incidence of thrombotic events in Japan are scarce [21,22]. In this study, 19 patients (24.4%) with MPNs had a history of thrombotic events, which is a similar to the ratio reported in Western studies [12], in which the prevalence of major thrombosis was 11.0–38.6% at the time of diagnosis and 8.0–30.7% during follow up [23,24].

Consistent with previous observations [16,17,25-27], our results showed that the WBC count tends to be higher in patients with the *JAK2* V617F mutation than in those without, but the difference was not statistically significant (p=0.131). In another study, subjects with increased leukocytes (≥10,000/μl) demonstrated a higher incidence of the *JAK2* V617F mutation than those with leukocytes <10,000/μl (p=0.046). These results show a significant association between the *JAK2* V617F mutation and increased leukocyte count (≥10,000). Hemoglobin levels and platelet counts, however, did not show significant differences.

Some studies have demonstrated that patients with *JAK2* V617F are more susceptible to thrombotic events [28]. Similarly, our data showed an association between the *JAK2* V617F mutation and thrombotic events (p= 0.047) in MPN patients. To assess the associations between other factors and TE, multivariate analysis was performed (data was not shown). However, after multivariate analysis no significant differences remained. Other factors, such as hypertension, and diabetes mellitus and hyperlipidemia, which are typically prevalent risk factors of TE, were not considered risk factors of TE in our study. It was suggested that the number of patients was too small to assess these risks by multivariate analysis. De Stefano V et al. [27,29,30] suggested that ET patients with an *JAK2* V617F allele burden >50% have a higher risk of recurrent thrombosis, although this could not be verified in our study (Table 3), possibly due to the smaller number of analyzed patients.

In patients with MPNs, the following mechanisms of thrombogenesis induced by *JAK2* V617F have been proposed: a higher hematocrit and whole-blood viscosity; enhanced platelet-endothelium interactions; formation of platelet-leukocyte aggregates and procoagulant microparticles; and increased monocyte tissue factor expression and production of proinflammatory cytokines, P-selectin, and mediators of cell damage [12,20,31-34]. In several clinical studies, thrombosis in MPN patients was thought to be related to leukocytosis rather than thrombocytosis [21]. Recently highlighted is the interaction between platelets and neutrophils at sites of vessel injury, hemorrhage, thrombosis, and inflammation. Neutrophils are bound by activated platelets, deplete the content of their granules, and express the procoagulant molecule, tissue factor. Neutrophils form complexes with platelets and endothelial cells mediated by increased expression of adhesion molecules, and fibrin generation is induced by tissue factors originating in monocytes and neutrophils. In addition, the presence of microbes and/or cytokines induces the formation of neutrophil extracellular traps and causes platelet adhesion, activation, aggregation, and subsequent thrombus formation [35].

In summary, the *JAK2* V617F mutation was analyzed as a useful and indispensable tool for assessing patients with MPNs. Patients with the *JAK2* V617F mutation were older (p=0.003) with an increased leukocyte count (p=0.046) compared to patients without the mutation. Remarkably, the *JAK2* V617F mutation was significantly associated with an increased risk of thrombotic events. In addition to standard risk factors, including age >60 years, a history of previous thrombosis, and leukocytosis [36], the *JAK2* V617F mutation should be further investigated to ascertain the thrombotic risk in patients with MPNs.

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