Influence of *JAK2*^{V617F} allele burden on phenotype in essential thrombocythemia

Elisabetta Antonioli, Paola Guglielmelli, Giada Poli, Costanza Bogani, Alessandro Pancrazzi, Giovanni Longo, Vanessa Ponziani, Lorenzo Tozzi, Lisa Pieri, Valeria Santini, Alberto Bosi, and Alessandro M. Vannucchi, for the Myeloproliferative Disorders Research Consortium (MPD-RC)

Department of Hematology, University of Florence, Florence, Italy; and the MPD-RC, Mount Sinai Hospital, New York, USA

Funding; this study was supported by Associazione Italiana per la Ricerca sul Cancro, Milano; Ente Cassa di Risparmio di Firenze; and MIUR (COFIN 2006067001_003) grants to AMV. AP was the recipient of a fellowship from the Associazione Italiana per le Leucemie, Firenze.

The project described was supported by grant number PO1CA108671 from the National Cancer Istitute. The content is solely the responsability of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institute of health.

Manuscript received April 26, 2007
Manuscript accepted September 5, 2007.

Correspondence:
Alessandro M. Vannucchi,
Department of Hematology,
University of Florence, 50134
Florence, Italy.
E-mail: amvannucchi@unifi.it

ABSTRACT

Background
Fifty to sixty percent of patients with essential thrombocythemia harbor the *JAK2*^{V617F} mutation. The impact of this mutation on clinical phenotype is still debated. The aim of this study was to evaluate possible correlations between *JAK2*^{V617F} mutant allele burden and both clinical presentation and hematologic abnormalities in essential thrombocythemia patients.

Design and Methods
In this single-center retrospective study, *JAK2*^{V617F} allele load was measured by sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in the granulocytes of 260 patients diagnosed as having essential thrombocythemia according to WHO criteria.

Results
Median V617F allele burden in patients with the mutation (n=165, 63.4%) was 24%, ranging from 1% to 87%; an allele burden greater than 51% was found in 5% of the patients. Older patients presented progressively higher percentages of the V617F allele. Signs of stimulated erythropoiesis and myelopoiesis, as well as higher *PRV-1* levels, were found in patients with the mutation, but no linear correlation with load of mutant allele could be ascertained; on the other hand, the frequency of patients with erythropoietin-independent erythroid colonies progressively increased depending on mutant allele load. Splenomegaly and microvessel symptoms were significantly more represented among patients with greater than 50% and 25% *JAK2*^{V617F} allele burden, respectively. Increasing mutant allele load correlated with higher frequency of arterial thrombosis at diagnosis, as confirmed also in multivariate analysis; the relative risk was 3.0 (95% CI 1.3-6.8; p=0.01) in patients having a greater than 25% mutant allele burden.

Conclusions
The JAK2^{V617F} mutant allele burden contributes to determining the clinical phenotype in patients with essential thrombocythemia.

Key words: *JAK2*^{V617F}, allele burden, essential thrombocythemia, phenotype

Citation: Antonioli E, Guglielmelli P, Poli G, Bogani C, Pancrazzi A, Longo G, Ponziani V, Tozzi L, Pieri L, Santini V, Bosi A, Vannucchi AM. Influence of JAK2V617F allele burden on phenotype in essential thrombocythemia. Haematologica. 2008 Jan; 93(1):41-48.
DOI: 10.3324/haematol.11653

Introduction

An acquired mutation in exon 14 of the Janus tyrosine kinase 2 gene (*JAK2*) leading to a valine→phenylalanine change at position 617 (*JAK2*^{V617F}) in the JH2 pseudokinase domain,[1-4] has been described in Philadelphia-negative chronic myeloproliferative disorders (MPD);[5,6] it can be found in approximately 95% cases of polycythemia vera (PV) and in approximately 50-60% cases of of essential thrombocythemia (ET) or primary myelofibrosis (PMF).[7] A role for the *gain-of-function*[8] of mutant *JAK2* in the pathogenesis of MPD is supported by *in vitro* studies, which showed growth factor independence and enhanced JAK-STAT signaling in cells transfected with the mutant allele, and by the results of *in vivo* animal models.[2,9,10] The *JAK2*^{V617F} mutation can be harbored in the heterozygous or homozygous state, the latter deriving from mitotic recombination;[1-4] homozygosity for this mutation is found in about 30% of PV and PMF patients but it is rare (2-4%) in ET.

In patients with the different MPD, the *JAK2*^{V617F} mutation has been variably associated with higher indices of erythropoiesis,[11,12] unchanged or decreased platelet counts,[11,12] greater occurrence of thrombosis,[4,13] bone marrow fibrosis or cytoreductive treatment,[4] older patients' age,[3,4,14,15] longer disease duration or poorer survival.[16] In particular, in the case of ET, it has been suggested that *JAK2*^{V617F} mutation imparts patients a *PV-like* phenotype;[11] furthermore, the risk of thrombosis has been found to be increased in ET patients with the mutation,[17] particularly in those harboring it in the homozygous state.[13] It is still unclear, however, how the presence of the *JAK2*^{V617F} mutation can help the clinician in identifying categories of patients with unique clinical and prognostic characteristics. One of the reasons underlying current uncertainties is that genomic techniques currently employed for detection of the *JAK2*^{V617F} mutation do not produce quantitative information about the level of the mutant allele, which might represent an important variable influencing disease presentation. The levels of expression of genes such as *PRV-1* and *NFE-2*, the number of CD34^{+} cells in the peripheral blood, and the concentration of alkaline leukocyte phosphatase (ALP), have all been shown to be dependent on mutant allele burden.[18-20] A prospective study in PV patients identified those with greater than 75% V617F allele as having more pronounced signs of myeloproliferation, a higher incidence of thrombosis and as being at greater risk of needing chemotherapy.[21]

In the current study we explored whether there was an association between burden of the *JAK2* mutation and clinical presentation in a retrospective series of 260 patients with ET. We correlated the levels of *JAK2*^{V617F} allele load in granulocytes with clinical and hematologic characteristics and with some markers typical of MPD, which included X-chromosome inactivation pattern (XCIP), *PRV-1* expression level and erythropoietin-independent erythroid colony (EEC) formation. Additionally, quantitative analysis of the *JAK2*^{V617F} mutation was performed serially in 35 patients during follow-up to evaluate any fluctuations in mutant allele load.

Design and Methods

Patients

This study involved 260 patients with a diagnosis of ET according to WHO criteria,[22] 115 of whom had been previously included in a multicenter GIMEMA study.[13] The design of this study was that of a retrospective, single center study; the only criteria necessary for inclusion were a diagnosis of ET satisfying the WHO criteria, complete chart records, and availability of a stored or fresh granulocyte DNA sample for *JAK2* mutational analysis. If patients had been first genotyped during follow-up, only those not receiving cytoreductive drugs were considered. All diagnoses were re-evaluated using the original clinical records and bone marrow biopsies by an *ad hoc* panel of four hematologists and two pathologists. The study was approved by the local Ethical Committee and informed consent was obtained from all patients included.

Clinical outcomes

The presence of splenomegaly was objectively determined on the basis of an ultrascan demonstrating an organ with a longitudinal diameter ≥12 cm. Major thrombotic events, whether arterial or venous (ischemic stroke, transient ischemic attack, myocardial infarction, angina pectoris, peripheral arterial thrombosis, deep vein thrombosis including cerebral and splanchnic venous thromboses, and pulmonary embolism) were recorded only if they happened after the diagnosis or in the preceding 2 years[23] and were objectively documented.[24] Only major bleeding events were considered, and included fatal hemorrhages, non-fatal intracranial bleeding or any other hemorrhages requiring surgery or causing a hemoglobin reduction of ≥2 g/dL and/or needing transfusion of two or more red blood cell units. The presence of symptoms due to microvessel disorder (headache, acral paresthesia, erythromelalgia, transient neurological and visual disturbances) was routinely investigated and recorded in the chart records by referring physicians, but for the purposes of this study they were considered only in the case that they had been described by the patient as non-occasional, of recent onset, and often ameliorated by aspirin.

Analysis of the JAK2^{V617F} mutation

Peripheral blood granulocytes were separated by differential centrifugation over a Ficoll-Paque gradient, and contaminating red cells were removed by hypotonic lysis; DNA was purified using the QIAmp DNA blood

kit (Qiagen, GmbH, Germany), and quantified with NanoDrop technology (Wilmington, DE, USA). All patients were routinely genotyped for the *JAK2*^{V617F} mutation by an allele-specific (ASO) polymerase chain reaction (PCR), using 75 ng granulocyte DNA. To evaluate whether the mutation was carried in the homozygous or heterozygous state, PCR products were digested with *Bsa*XI restriction enzyme (New England Biolabs, Hitchin, UK) as described by Baxter *et al.*[1] The mutant allele burden was measured by a quantitative real time (QRT)-PCR assay, using 20 ng genomic DNA. PCR amplification and detection were performed on an ABI Prism 7300 analyzer (Applied Biosystems) using the following cycling conditions: 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Primers flanking the mutant region (forward primer 5'-AAGCTTTCTCACAAGCATTTGGTTT-3'; reverse primer 5'-AGAAAGGCATTAGAAAGCCTG TAGTT-3') were employed together with Taqman probes which were specific for either the wild type (VIC-5'-TCTC-CACAGACACATAC-3'MGB) or the mutant *JAK2* allele (FAM-5'- TCCACAGAAACATAC-3'-MGB). All samples were analyzed in triplicate and the amount of *JAK2*^{V617F} allele was calculated by comparison with serial dilutions of mutant DNA, obtained from a PV patient with 100% mutant alleles, and wild-type DNA from healthy subjects. The mean of triplicate ΔCT determinations ($C_T^{JAK2V617F}$–C_T^{JAK2WT}) was used to calculate the percentage of mutant alleles. Positive and negative controls were included in each assay; inter- and intra-assay variation was 3% and 5%, respectively.

Other biological markers

PRV-1 expression level. The level of expression of neutrophil *PRV-1*, and of *GAPDH* as the housekeeping gene, was quantified using the Taqman one-step procedure, as described previously.[25] The *PRV-1* cycle threshold (C_T) ratio was calculated from the mean value of triplicate *PRV-1* C_T determinations divided by the mean of triplicate *GAPDH* C_T; the *PRV-1* C_T ratio in healthy blood donors (n=30) was 1.37±0.09; *PRV-1* over-expression was defined in the presence of a *PRV-1/GAPDH* C_T ratio ≤1.17.

Erythropoietin-independent erythroid colonies. *In vitro* assays for EEC was performed by plating 2.5×10^5 peripheral blood mononuclear cells in Methocult H4531 medium (StemCell, Vancouver, Canada) in the absence of added erythropoietin. Concurrent plates containing optimal amounts of erythropoietin (2 U/mL) were prepared as control cultures. Dishes were incubated in a humidified atmosphere of 5% CO_2 at 37°C, and hemoglobinized colonies were enumerated on day 14 by standard criteria.

Clonal hematopoiesis. XCIP evaluation was performed at the HUMARA locus in female patients aged less than 60 years old using a PCR assay and enzymatic digestion with RsaI and HpaII, as described elsewhere.[26] Immunomagnetically purified $CD3^+$ T-lymphocytes were used as control cells.

Statistical analysis

Data were processed using the GraphPad InStat (GraphPad Software, San Diego, CA, USA) and the SPSS (Statsoft Inc, Tulsa, OK, USA) software. We used the χ^2 or Fisher's exact test (two by two table) or χ^2 test for trend (larger contingency table), as appropriate, to compare categorical variables among the groups which had been categorized according to mutation load. The analysis of continuous variables among the groups was performed using the Mann-Whitney U test or Kruskal-Wallis test with Dunn's method in the case of multiple comparisons. Spearman's rank non-parametric correlation test was performed to analyze correlations between *JAK2*^{V617F} allele burden and hematologic parameters. Unconditional logistic regression models with backward stepwise analysis were used to identify factors that were associated with an increased probability of having thrombotic events. A p value of less than 0.05 was considered to indicate statistical significance; all tests were two-tailed.

Results

Characteristics of the patients

We studied 260 subjects in whom a diagnosis of ET was made based on WHO criteria; their clinical characteristics and hematologic parameters at diagnosis, as well as major clinical events associated with disease, are reported in Table 1. Their median age was 52 years (range, 16-93). Of these 260 subjects, 195 (75%) were females, making the proportion of females higher than that in most published series of ET; this was due to trivial, uncontrolled reasons, and was not the result of a selection. At diagnosis, 72 patients (28%) had splenomegaly and in eight patients (3%) the spleen diameter was greater than 15 cm. Twenty-three patients (9%) reported aquagenic pruritus and 19 (7%) presented with systemic symptoms. One hundred and sixty-one patients (62%) required cytoreductive treatment, either because they were high-risk patients, according to current criteria,[27] or because signs and/or symptoms of myeloproliferation needed to be controlled. Fifty-six patients (21%) had major thromboses, 26 of which were detected at diagnosis or had occurred within the preceding 2 years, and 25 during follow-up; five patients had a second thrombotic event during follow-up. A detailed description of the thrombotic events is presented in Table 1. Microvessel symptoms were reported by 73 patients (28%). Hemorrhages affected 15 patients (6%), and in all cases were gastrointestinal bleeds; seven of these occurred at the time of diagnosis. The median follow-up was 36 months (range, 1-120); during this period four patients died of causes unrelated to their hematologic disease, one patient developed myelofibrosis and two still-alive patients developed solid tumors.

JAK2^{V617F} mutational burden

The median time from diagnosis to blood sampling for mutational analysis was 2.0 years (range, 0-3). DNA samples for mutational analysis were collected at diagnosis in 102 patients (39%), within 1 year from diagnosis in 80 (31%), between 1 and 2 years from diagnosis in 50 (19%), while the remaining 28 patients (11%) were genotyped from 2 to 3 years after diagnosis. According to ASO PCR, 157 patients (60%) carried *JAK2*^{V617F} mutations, of whom 151 (96%) were considered heterozygous and six (4%) homozygous, accounting for 58% and 2% of the entire patient population, respectively. However, by using QRT-PCR, the frequency of patients with the mutation rose to 63% (n=165) and the percentage of those displaying >51% mutant alleles was 5%. The median *JAK2* mutant allele ratio in these 165 patients with the mutation was 24% (range, 1-87%), which is significantly lower than the level we previously found in PV patients (n=173, 52% range 1-100%)[21] or in patients with PMF (n=55; 8%: range, 1-100%) or secondary forms of myelofibrosis (n=20; 61%: range, 1-100%) (p<0.001 for all) (Figure 1). *JAK2*^{V617F} levels ranged from 1% to 25% in 101 patients (39%), from 26% to 50% in 51 patients (20%), from 51% to 75% in 10 patients (4%) while only three patients (1%) had *JAK2*^{V617F} levels greater than 75% (Figure 1).

Correlation of *JAK2*^{V617F} burden with hematologic parameters

Patients with the *JAK2*^{V617F} mutant allele had significantly higher leukocyte counts (p=0.02), hemoglobin concentration (p<0.0001), hematocrit (p<0.0001), alkaline phosphate level (p=0.04) and lower platelet count (p=0.03), as compared to wild-type patients (Table 1). However, there was no clear dose-dependent correlation between these hematologic parameters, considered either at diagnosis or at the time of blood sampling, and the burden of *JAK2* mutant allele (*not shown in detail*).

EEC formation was detected in 52% of evaluated patients (n=113), accounting for 40% and 60% of *JAK2*^{V617F} wild-type and mutant patients, respectively (p=0.05). We observed a significant correlation between EEC and load of *JAK2* mutant allele (p=0.0019) (Table 2); indeed, all 13 patients with more than 50% mutant alleles displayed EEC formation as compared to half of those with 1-25% V617F alleles. XCIP analysis was performed in 122 patients, and was informative in 104 (85%); 11 females were excluded because of constitutional skewing and seven because of homozygosity at the HUMARA locus. Sixty-five per cent of informative females had clonal hematopoiesis. The frequency of patients with clonal hematopoiesis was similar among those with wild-type (68%) or mutant *JAK2*^{V617F} (63%); furthermore, there was no difference in *JAK2*^{V617F} mutant allele load between patients with clonal or polyclonal hematopoiesis (13% in both groups). Finally, we found no significant correlation between *PRV-1* expression

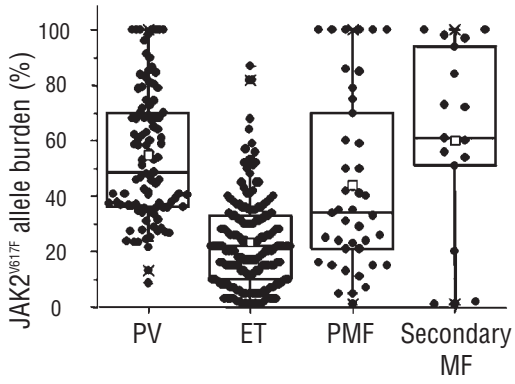

Figure 1. ***JAK2***^{V617F} **allele burden in the 165 patients with the *JAK2* mutation included in the study. For comparison, results from patients with PV (n=135)[44], primary myelofibrosis (PMF) (n=55) or secondary forms of myelofibrosis (Post-MF) (n=20) are presented. The level found in ET patients was significantly lower than the levels in all other MPD categories (p<0.001). Boxes represent the interquartile range that contains 50% of the subjects, the small square inside the mean value, and the horizontal line inside marks the median; the bars show the upper and lower range of values.**

Table 1. Hematologic and clinical features at diagnosis in the 260 patients with ET considered in the study.

		JAK2^{V617F}		
	All patients (n=260)	*Wild-type pts (n=95)*	*Mutated pts (n=165)*	*p*
Male/female	65/195	26/69	39/126	NS
Disease duration (months)°	36 (1-120)	39 (1-120)	30 (1-120)	NS
Age (yrs)°	52 (16-93)	50 (16-87)	55 (19-93)	=0.005
Hematologic parameters				
Hematocrit (%)§	42±4	41±4	43±4	<0.0001
Hemoglobin (gr/dL)§	14.0±1.4	13.4±1.3	14.4±1.3	<0.0001
MCV (fL)§	87±4	88 ± 6	87±8	NS
White cell count (x10^9/L)§	9.3±3.1	8.8±3.0	9.5±3.2	=0.02
Platelet count (x10^9/L)§	880±314	959±380	835±260	=0.03
Lactate dehydrogenase (U/L)§	351±167	347±137	353±180	NS
ALP (Arbitrary Units)§	130±54	111±45	138±56	=0.04
Clinical characteristics				
Splenomegaly (%)	72 (28%)	23 (24%)	49 (29%)	NS
Splenomegaly >15 cm (%)	8 (3%)	1 (1%)	7 (4%)	NS
Pruritus (%)	23 (9%)	9 (9%)	14 (8%)	NS
Systemic symptoms (%)	19 (7%)	6 (6%)	13 (8%)	NS
Cytoreductive therapy requirement (%)	161 (62%)	56 (59%)	105 (63%)	NS
Patients with major thrombotic events (%)	56 (21%)	16 (17%)	40 (24%)	NS
Patients with major arterial thrombosis (%)	30 (11%)	8 (8%)	22 (13%)	NS
Patients with major venous thrombosis (%)	26 (10%)	8 (8%)	18 (11%)	NS
Patients with thrombosis at diagnosis (%)	31 (12%)	8 (8%)	23 (14%)	NS
Patients with thrombosis during follow-up (%)	25 (10%)	8 (8%)	17 (10%)	NS
Patients with microvessels disease (%)	73 (28%)	18 (19%)	55 (33%)	=0.01
Patients with major hemorrhages (%)	15 (6%)	4 (4%)	11 (6%)	NS

°:Median value (range) is reported; §: mean value (±SD) is reported. NS: not statistically significant. MCV: mean corpuscular volume; ALP: alkaline phosphatase.

level and *JAK2*^{V617F} allele burden, although the level of *PRV-1* was significantly lower in ET patients with the wild-type genotype than in the ET patients with the mutation (1.23±0.15 and 1.14±0.12, respectively; p=0.03) (Table 2). In 74 patients, the three tests (EEC, *PRV-1* and XCIP) were concurrently evaluated; overall concordance was found in only 25% of the cases, which comprised nine and ten patients who had all of the tests positive or negative, respectively.

Correlation of *JAK2*^{V617F} burden with clinical characteristics

Patients with the *JAK2*^{V617F} mutation were older than those with wild-type alleles (p=0.005), and the mutant allele load progressively increased in older patients (Figure 2) (p<0.0001; 95% CI 0.16-0.44, Spearman's rank test). In contrast, the median disease duration was inversely related to patients' age: it was 42, 60, 27 and 18 months in patients less than 50 years old, 51-60 and 61-70 years old, or older than 70 years, respectively (p<0.001).

Splenomegaly was equally present among patients with or without the *JAK2*^{V617F} mutation (29% and 24%, respectively; p=0.8); however, median allele burden was significantly higher (27% versus 20% p=0.04) in patients who had splenomegaly as well as in those with a larger spleen (30% versus 20%, p=0.03). Furthermore, there was a correlation between greater *JAK2*^{V617F} allele burden and the frequency of patients who had either splenomegaly (p=0.03, χ^2 test for trend; Figure 3) or a larger spleen (p=0.02, χ^2 test for trend). The relative risk (RR) of having splenomegaly was 2.2 (95% CI 1.2-4.1, p=0.03) in the presence of greater than 50% V617F allele using patients with wild-type alleles as the reference population.

There was no difference between patients with mutations or wild-type alleles as regards the frequency of total (arterial plus venous) thrombotic events, which affected 24% and 17% of the patients, respectively (p=0.3), either considering thrombosis at diagnosis (14% and 8%) or in the follow-up (10% and 8%, respectively). The mutant allele burden was similar in patients who did or did not have thrombosis (21% and 22%, respectively). However, when arterial and venous events were considered separately, it was found that allele burden was significantly higher in patients with arterial thrombosis at diagnosis (33% versus 20%; p=0.005), and that the frequency of patients with thrombosis progressively increased with greater allele burden (p=0.003, χ^2 test for trend). The RR for arterial thrombosis at diagnosis in patients with greater than 25% mutant allele was 3.0 (95% CI, 1.3-6.8; p=0.01). The impact of V617F mutant allele burden on arterial thrombosis at diagnosis was mantained in multivariate analysis, which included age, leukocyte count, hemoglobin and platelet count as co-variates (p=0.04). On the other hand, there was no difference between patients with mutant and wild-type alleles as regards the rate with venous events (Table 1) nor there was any meaningful effect of allele burden (*not shown in detail*).

Symptoms due to microvessel involvement affected patients with and without *JAK2*^{V617F} mutations similarly (26% and 20%, respectively; p=0.27); however, when patients were categorized according to their V617F allele burden, the RR of suffering symptoms from microvessel disease increased progressively (p=0.0008, χ^2 test for trend; Figure 3), being 2.2 (95%CI 1.3-3.8; p=0.03) and 2.4 (95% CI 1.2-5.0; p=0.03) in patients with a mutant

Table 2. Relationships between *JAK2*^{V617F} allele burden and percentage of patients with abnormal results of laboratory assays.

	Wild-type	*JAK2*^{V617F}	p=	*JAK2*^{V617F} *allele burden*			$p^{\S}$=
				1-25%	*26-50%*	*51-100%*	
EEC+^{VE} (59/105)	40% (18/45)	60% (41/60)	0.05	52% (19/36)	81% (9/11)	100% (13/13)	0.0019
XCIP+^{VE} (68/104)	68% (30/44)	63% (38/60)	NS	62% (30/48)	70% (7/10)	50% (1/2)	NS
PRV-1+^{VE} (63/138)	40% (21/52)	49% (42/86)	NS	47% (32/67)	46% (6/13)	66% (4/6)	NS
PRV-1 Levels	1.23 ±0.15	1.14 ±0.12	0.03	1.14 ±0.12	1.16 ±0.10	1.08 ± 0.16	NS

EEC+^{VE} indicates patients showing erythropoietin-independent erythroid colony formation; XCIP+^{VE} indicate females presenting clonal hematopoiesis based on the HUMARA assay; for the definition of PRV-1 over-expression, refer to the Design and Methods section. The total number of patients evaluated was 105, 104, and 138, respectively. The number of patients positive for each marker over total number of patients analyzed is shown within brackets in each row. §the p values indicates difference among the three categorized groups of patients with the V617F mutation according to the analysis of trend (χ^2 test for trend or Kruskal-Wallis test with the use of Dunn's method for multiple comparisons, as appropriate).
NS: not statistically significant.

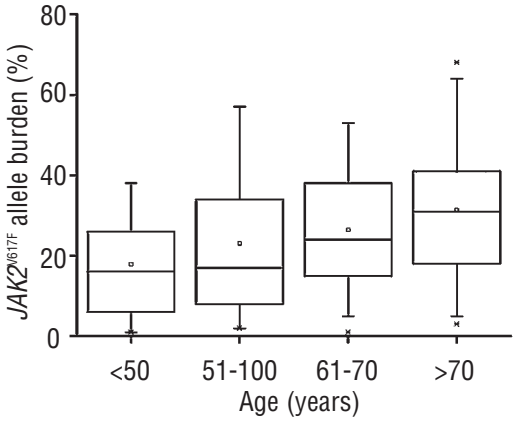

Figure 2. *JAK2*^{V617F} allele burden in different age groups. The 165 patients with *JAK2* mutation were divided in four age classes, as indicated. The age classes comprised 67, 29, 33, and 36 patients. The *JAK2* mutant allele burden in the two oldest age classes was significantly different from that measured in patients less than 60 years old (p=0.01 for the 61-70 year old class, and p=0.0006 for the >70 year old age class). Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line inside marks the median, the small square inside the mean value, and bars show the upper and lower range of values.

allele burden greater than 25% and 50%, respectively.

Finally, the mutant allele burden had no impact on pruritus, systemic symptoms, need for cytoreductive treatment, duration of disease or occurrence of hemorragic events (*not shown in detail*).

Kinetics of JAK2^{V617F} allele burden

Changes in *JAK2*^{V617F} allele level measured at diagnosis were evaluated in 35 patients for whom at least one additional sample, taken during follow-up over a median time of 18 months (range, 6-36 months), was available. None of the 20 patients who had *JAK2*^{V617F} wild-type alleles at diagnosis became positive for the mutation. Twelve out of the 15 patients with the mutation maintained stable V617F allele levels during follow-up, the median difference among samples being 5% (range, 1-12%). On the other hand, three patients showed overt changes in their mutant allele burden; in two of them V617F allele levels decreased from 22% and 29% to 4% and 3%, respectively, during treatment with hydroxyurea. However, an additional four patients receiving hydroxyurea did not show appreciable changes of allele burden over time. The third patient presented an increase from 82% to 100% of V617F allele in spite of cytoreductive treatment with hydroxyurea and a purine analog, which was instituted because of progressive leukocytosis with erythroblastosis, accompanied by the onset of transfusion-dependent anemia, suggestive of transformation to post-thrombocythemic myelofibrosis (the patient refused to undergo a bone marrow biopsy).

Discussion

Among the chronic myeloproliferative disorders, ET is characterized by greatest heterogeneity both as regards its clinical profile and at the cellular and molecular levels, since at least one third of females with ET have apparently polyclonal hematopoiesis,[28-32] although there may be pitfalls in the interpretation of clonality assays;[33] in addition, only 50 to 60% of patients have the *JAK2*^{V617F} mutation, while a minority (1%) harbor *MPL* mutations.[34] Current data about the prognostic relevance of the *JAK2*^{V617F} mutation in patients with ET are still partially inconclusive. The presence of the mutation has been associated with venous thrombosis occurring in the years before diagnosis in a large series,[11] while in other studies no such correlation was found.[12,35] Furthermore, in a comparative analysis of patients with ET and PV, ET patients with mutations had significantly more thrombotic events than their wild-type counterpart, and did not differ under this respect from PV patients.[17] Intriguingly, the *JAK2*^{V617F} mutation was recently reported as being an independent risk factor for fetal loss in ET females.[36] Finally, in a large multicenter study, ET patients harboring homozygous *JAK2*^{V617F} mutation had a 3.9-fold higher risk of major cardiovascular events as compared to both their heterozygous and wild-type counterparts.[13]

In this study, we employed a quantitative RT-PCR assay to measure *JAK2*^{V617F} alleles in 260 ET patients with the aim of evaluating whether the mutant allele load affected hematologic and clinical phenotype, possibly overcoming limitations of the qualitative assays that had been employed in previous studies. We were prompted to do this by our recent study in PV patients which showed that the extent of hematologic abnormalities and of some major clinical manifestations, in particular thrombosis and chemotherapy need, were actually dependent on the burden of V617F allele measured in granulocytes collected at diagnosis.[21] However,

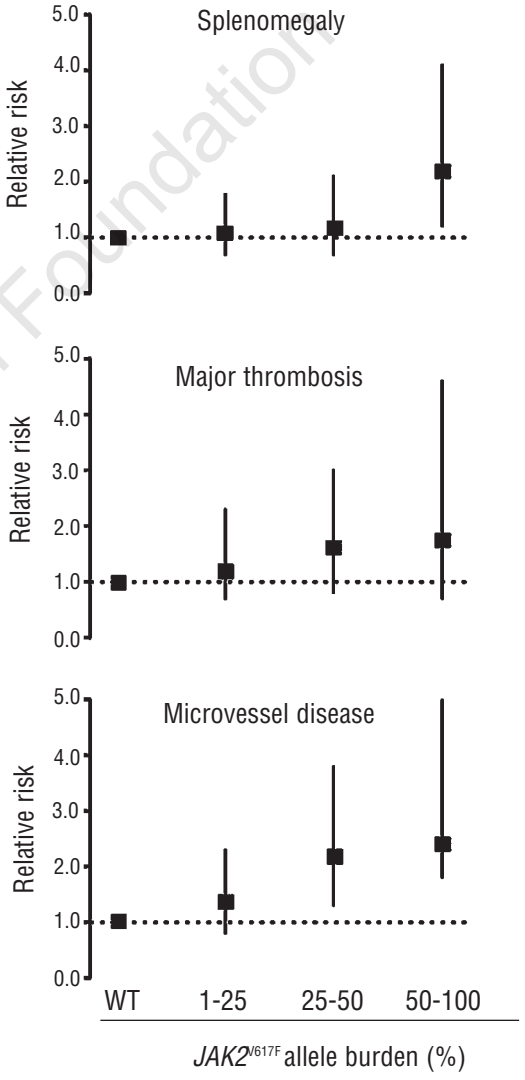

Figure 3. Effect of *JAK2*^{V617F} allele burden on clinical outcomes in ET patients. The analysis was performed to calculate the relative risk of a patient presenting with splenomegaly (upper panel), developing major thrombotic events (middle panel) or having microvessel disease (lower panel) according to the amount of *JAK2* mutant allele. Squares indicate the relative risk along with the 95% confidence interval (vertical bars). The reference category was patients who were wild-type (WT) for the mutation.

another study in PV patients from the Mayo Clinic found no significant correlation between allele burden and clinical manifestations;[37] a different source of DNA (bone marrow instead of granulocytes) and the fact that patients in this study were examined variably during follow-up rather than at diagnosis might underlie these discrepancies.

Consistent with previous data, ET patients with *JAK2*^{V617F} mutation had higher erythrocyte and leukocyte counts, and reduced platelet levels; however, the extent of these abnormalities was not significantly dependent on the burden of mutant alleles. Similar findings were reported in a recent paper by Tefferi's group, in which 96 ET patients with mutations were analyzed using a quantitative assay.[38] Intriguingly, these results differ from those we reported for PV patients,[21] in whom abnormalities in hematologic parameters were dose-dependently correlated with V61F allele burden. We hypothesize that this different behavior might be accounted for by the significantly lower burden of mutant alleles actually present in the ET patients (Figure 1), which prevented a direct cause-effect relationship from being revealed. This statement is indirectly supported by the finding that the percentage of ET patients displaying EEC formation (a phenomenon which is a very sensitive indicator of autonomous activation of the JAK-STAT pathway)[39] progressively increased depending on the amount of mutant allele. Also, no correlation was found between either the presence or burden of V617F allele with clonal hematopoiesis by XCIP analysis[40] or *PRV-1* expression level.[41] Finally, patients with the *JAK2*^{V617F} mutation were older than those with wild-type alleles,[11,13,15] and the load of mutant allele progressively increased with age.

Confirming previous observations,[42] we found that none of patients who had wild-type alleles at diagnosis later became positive, while we report some decrease of allele burden in three out of seven patients who were receiving hydroxyurea treatment. Given the low number of patients evaluated, we cannot draw any firm conclusions from these findings, which might have been non-specific unlike those reported in PV patients receiving interferon-α.[43]

The categorization of subjects according to the load of V617F allele measured by QRT-PCR in their granulocytes allowed us to reveal some effects that had been otherwise masked by the analysis of mutated versus wild-type patients considered as a whole. In fact, we observed that splenomegaly was significantly more frequent in patients having greater than 50% of the mutant allele, while symptoms due to microvessel disease were over-represented when the mutant allele level was just over 25%. Furthermore, we found that there was a 3-fold greater risk of arterial thrombosis at diagnosis in patients with more than 25% V617F allele, even though the difference between patients with the mutation and wild-type patients all together had not attained statistical significance. At variance with other studies, we found no association between the presence of mutant alleles and venous events,[11] but this is likely because of the low number of subjects who had venous thrombosis in our series.

Overall, these observations in ET add to the growing idea that hematologic abnormalities, as well as defined aspects of clinical phenotype, in MPD patients are at least partially related to the relative representation of the V617F allele in the context of normal hematopoiesis; this would also help to explain why *JAK2*^{V617F}-associated abnormalities are seen more clearly in patients with PV[21,45] or primary myelofibrosis,[16] whose median allele burden is substantially greater than that of ET patients. However, whether *JAK2*^{V617F} mutant status and/or allele burden might be fruitfully employed as novel criteria for risk stratification in patients with ET requires controlled prospective trials.

Authorship and Disclosures

EA and PG performed the research, evaluated the data and collaborated in writing the manuscript; GP, CB, AP, VP, LT, LP performed the molecular assays; VS, GL, AB and AMV contributed patients and evaluated clinical records; AMV designed the research, analyzed the data and wrote the manuscript.

The authors reported no potential conflicts of interest.

References

1. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005; 365: 1054-61.
2. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005;434: 1144-8.
3. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387-97.
4. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779-90.
5. Dameshek W. Some speculations on the myeloproliferative syndromes. Blood 1951;6:372-5.
6. Spivak JL. Diagnosis of the myeloproliferative disorders: resolving phenotypic mimicry. Semin Hematol 2003; 40:1-5.
7. Mesa RA, Verstovsek S, Cervantes F, Barosi G, Reilly JT, Dupriez B, et al. Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the international working group for myelofibrosis research and treatment (IWG-MRT). Leuk Res 2007;31:737-40.
8. Cazzola M, Skoda R. Gain of function, loss of control - a molecular basis for chronic myeloproliferative disorders. Haematologica 2005; 90: 871-4.

9. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood 2006;107:4274-81.
10. Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S, et al. Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. PLoS ONE 2006;1:e18.
11. Campbell PJ, Scott LM, Buck G, Wheatley K, East CL, Marsden JT, et al. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. Lancet 2005;366: 1945-53.
12. Antonioli E, Guglielmelli P, Pancrazzi A, Bogani C, Verrucci M, Ponziani V, et al. Clinical implications of the JAK2 V617F mutation in essential thrombocythemia. Leukemia 2005; 19:1847-9.
13. Vannucchi AM, Antonioli E, Guglielmelli P, Rambaldi A, Barosi G, Marchioli R, et al. Clinical profile of homozygous JAK2V617F mutation in patients with polycythemia vera or essential thrombocythemia. Blood 2007;110:840-6.
14. Kralovics R, Teo SS, Buser AS, Brutsche M, Tiedt R, Tichelli A, et al. Altered gene expression in myeloproliferative disorders correlates with activation of signaling by the V617F mutation of Jak2. Blood 2005;106:3374-6.
15. Wolanskyj AP, Lasho TL, Schwager SM, McClure RF, Wadleigh M, Lee SJ, et al. JAK2 mutation in essential thrombocythaemia: clinical associations and long-term prognostic relevance. Br J Haematol 2005;131:208-13.
16. Campbell PJ, Griesshammer M, Dohner K, Dohner H, Kusec R, Hasselbalch HC, et al. V617F mutation in JAK2 is associated with poorer survival in idiopathic myelofibrosis. Blood 2006;107:2098-100.
17. Finazzi G, Rambaldi A, Guerini V, Carobbo A, Barbui T. Risk of thrombosis in patients with essential thrombocythemia and polycythemia vera according to JAK2 V617F mutation status. Haematologica 2007;92: 135-6.
18. Vannucchi AM, Guglielmelli P, Antonioli E, Mappa S, Pancrazzi A, Bogani C, et al. Inconsistencies in the association between the JAK2 mutation and PRV-1 over-expression among the chronic myeloproliferative diseases. Br J Haematol 2006; 132:652-4.
19. Vannucchi AM, Pancrazzi A, Bogani C, Antonioli E, Guglielmelli P. A quantitative assay for JAK2(V617F) mutation in myeloproliferative disorders by ARMS-PCR and capillary electrophoresis. Leukemia 2006;20: 1055-60.
20. Passamonti F, Rumi E, Pietra D, Della Porta MG, Boveri E, Pascutto C, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood 2006;107:3676-82.
21. Vannucchi AM, Antonioli E, Guglielmelli P, Longo G, Pancrazzi A, Ponziani V, et al. Prospective identification of high-risk polycythemia vera patients based on JAK2V617F allele burden. Leukemia 2007;21:1952-9.
22. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292-302.
23. Policitemia GIS. Polycythemia vera: the natural history of 1213 patients followed for 20 years. Ann Intern Med 1995;123:656-64.
24. Marchioli R, Finazzi G, Landolfi R, Kutti J, Gisslinger H, Patrono C, et al. Vascular and neoplastic risk in a large cohort of patients with polycythemia vera. J Clin Oncol 2005;23: 2224-32.
25. Klippel S, Strunck E, Temerinac S, Bench AJ, Meinhardt G, Mohr U, et al. Quantification of PRV-1 mRNA distinguishes polycythemia vera from secondary erythrocytosis. Blood 2003;102:3569-74.
26. Vannucchi AM, Grossi A, Pancrazzi A, Antonioli E, Guglielmelli P, Balestri F, et al. PRV-1, erythroid colonies and platelet Mpl are unrelated to thrombosis in essential thrombocythaemia. Br J Haematol 2004;127:214-9.
27. Finazzi G, Barbui T. Risk-adapted therapy in essential thrombocythemia and polycythemia vera. Blood Rev 2005;19:243-52.
28. Chiusolo P, La Barbera EO, Laurenti L, Piccirillo N, Sora F, Giordano G, et al. Clonal hemopoiesis and risk of thrombosis in young female patients with essential thrombocythemia. Exp Hematol 2001;29:670-6.
29. el Kassar N, Hetet G, Li Y, Briere J, Grandchamp B. Clonal analysis of haemopoietic cells in essential thrombocythaemia. Br J Haematol 1995;90:131-7.
30. Harrison CN, Gale RE, Machin SJ, Linch DC. A large proportion of patients with a diagnosis of essential thrombocythemia do not have a clonal disorder and may be at lower risk of thrombotic complications. Blood 1999;93:417-24.
31. Shih LY, Lin TL, Dunn P, Wu JH, Tseng CP, Lai CL, et al. Clonality analysis using X-chromosome inactivation patterns by HUMARA-PCR assay in female controls and patients with idiopathic thrombocytosis in Taiwan. Exp Hematol 2001;29:202-8.
32. Shih LY, Lin TL, Lai CL, Dunn P, Wu JH, Wang PN, et al. Predictive values of X-chromosome inactivation patterns and clinicohematologic parameters for vascular complications in female patients with essential thrombocythemia. Blood 2002;100: 1596-601.
33. Chen GL, Prchal JT. X linked clonality testing: interpretation and limitations. Blood 2007;110:1411-9.
34. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med 2006;3:e270.
35. Wolanskyj AP, Lasho TL, Schwager SM, McClure RF, Wadleigh M, Lee SJ, et al. JAK2 mutation in essential thrombocythaemia: clinical associations and long-term prognostic relevance. Br J Haematol 2005;131:208-13.
36. Passamonti F, Randi ML, Rumi E, Pungolino E, Elena C, Pietra D, et al. Increased risk of pregnancy complications in patients with essential thrombocythemia carrying the JAK2(V617F) mutation. Blood 2007; 110:485-9.
37. Tefferi A, Strand JJ, Lasho TL, Knudson RA, Finke CM, Gangat N, et al. Bone marrow JAK2V617F allele burden and clinical correlates in polycythemia vera. Leukemia 2007; 21:2074-5.
38. Kittur J, Knudson RA, Lasho TL, Finke CM, Gangat N, Wolanskyj AP, et al. Clinical correlates of JAK2V617F allele burden in essential thrombocythemia. Cancer 2007; 109: 2279-84.
39. Dupont S, Masse A, James C, Teyssandier I, Lecluse Y, Larbret F, et al. The JAK2 V617F mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera. Blood 2007; 110: 1013-21.
40. Antonioli E, Guglielmelli P, Pancrazzi A, Bogani C, Verrucci M, Ponziani V, et al. Clinical implications of the JAK2 V617F mutation in essential thrombocythemia. Leukemia 2005; 19:1847-9.
41. Vannucchi AM, Guglielmelli P, Antonioli E, Mappa S, Pancrazzi A, Bogani C, et al. Inconsistencies in the association between the JAK2(V617F) mutation and PRV-1 over-expression among the chronic myeloproliferative diseases. Br J Haematol 2006;132:652-4.
42. Campbell PJ, Baxter EJ, Beer PA, Scott LM, Bench AJ, Huntly BJ, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood 2006;108:3548-55.
43. Kiladjian JJ, Cassinat B, Turlure P, Cambier N, Roussel M, Bellucci S, et al. High molecular response rate of polycythemia vera patients treated with pegylated interferon alpha-2a. Blood 2006;108:2037-40.
44. Vannucchi AM, Antonioli E, Guglielmelli P, Pancrazzi A, Bogani C, Pieri L, et al. Influence of the JAK2V617F mutational load on major clinical aspects in patients with polycythemia vera. Blood 2006;108:5A.
45. Tefferi A, Lasho TL, Schwager SM, Strand JS, Elliott M, Mesa R, et al. The clinical phenotype of wild-type, heterozygous, and homozygous JAK2(V617F) in polycythemia vera. Cancer 2006;106:631-5.