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Phenotypic variability within the JAK2 V617F-positive MPD: The roles of progenitor cell and neutrophil allele burdens

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Abstract

(1) Objective—The myeloproliferative disorders (MPD), polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF) differ phenotypically but share the same JAK2^{V617F} mutation. We examined the relationship of the quantitative JAK2^{V617F} allele burden to MPD disease phenotype among the three MPD classes and within PV.

(2) Methods—We measured the JAK2^{V617F} allele percentage in genomic DNA from neutrophils, CD34⁺ cells, and cloned progenitors in 212 JAK2^{V617F}–positive MPD patients and correlated the allele burdens to both disease class and disease features.

(3) Results—In ET and PV, the mean CD34⁺ cell JAK2^{V617F} allele burdens were lower than the corresponding neutrophil allele burdens, but these were equivalent in PMF. JAK2^{WT} progenitors were present in ET and PV when the CD34⁺ JAK2^{V617F} allele burden was lower than the neutrophil allele burden, but not in PV and PMF subjects in whom the CD34⁺ cell and neutrophil allele burdens were similar. CD34⁺ cell JAK2^{V617F} clonal dominance, defined as coherence between the CD34⁺ cell and neutrophil JAK2^{V617F} allele burdens, was present in 24% of ET, 56% of PV and 93% of PMF patients, and was independent of the CD34⁺ cell JAK2^{V617F} genotype. Clonally-dominant PV patients had significantly longer disease durations, higher white cell counts and larger spleens than nondominant PV patients.

(4) Conclusions—We conclude that the extent of JAK2^{V617F} CD34⁺ cell clonal dominance is associated with disease phenotype within the MPD, and in PV, is associated with extramedullary disease, leukocytosis and disease duration.

Introduction

Polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocytosis (ET) are clonal myeloproliferative disorders that share in common origin in a multipotent hematopoietic progenitor cell(1), cytogenetic abnormalities of chromosomes 1, 8, 9, 13 and 20(2), and growth factor-independent in vitro hematopoietic colony formation(3). Taken together, these shared phenotypic and genotypic features suggest that PV, PMF and ET might not be separate disorders but rather, different manifestations of the same disease. The recent discovery of the same JAK2 activating mutation (JAK2^{V617F}) in all three disorders supported this contention (4–6).

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However, PV, ET and PMF each have a unique epidemiology and natural history. Moreover, despite the same genetic lesion, the quantitative JAK2^{V617F} neutrophil and platelet allele burdens are variable due to gene dosage in individual cells and the fact that many JAK2^{V617F}-positive myeloproliferative disorder (MPD) patients retain JAK2 wild-type clones (JAK2^{WT})(7–10). Clonal dominance with suppression of normal hematopoietic progenitors is a feature of the MPD(7) and the extent of this could be responsible for the different clinical manifestations of PV, ET and PMF. To examine this possibility we analyzed the relationship of JAK2^{V617F} allele burden measured in peripheral blood CD34⁺ cells, cloned CD34⁺ colonies and neutrophils to clinical phenotype in PV, PMF and ET. We observed significantly lower neutrophil and CD34⁺ cell JAK2^{V617F} allele burdens in ET compared to either PV or PMF. Importantly, we also observed that in ET patients and many PV patients, the CD34⁺ cell JAK2^{V617F} allele burdens were lower than the corresponding neutrophil allele burdens, and that this was due to the presence of JAK2^{WT} CD34⁺ progenitor cells. In contrast, in PMF, CD34⁺ cell and neutrophil JAK2^{V617F} allele burdens were similar, and clonal dominance of JAK2^{V617F}-positive CD34⁺ cells was the rule.

The presence of wild-type CD34⁺ progenitor cells was associated with less extramedullary disease regardless of disease class, and in PV, was associated with shorter disease duration. Importantly, in PV the CD34⁺ cell JAK2^{V617F} allele burden correlated better with disease duration, leukocytosis and extramedullary disease than did the neutrophil JAK2^{V617F} allele burden. These data support a model of clonal expansion of JAK2^{V617F}-positive CD34⁺ progenitor cells that distinguishes ET and PV from PMF. Within PV, clonal dominance at the CD34⁺ progenitor cell level is time-dependent and associated with extramedullary disease.

Methods

Subjects

The study protocol was approved by our Joint Committee on Clinical Investigation and written informed consent was obtained from each patient. The diagnosis of PV and ET were based on the Polycythemia Vera Study Group criteria and included an elevated red cell mass obtained at the time of diagnosis or follow-up as indicated (11). The diagnosis of PMF(12) was based on the Italian Consensus Conference (13). The patients recruited to this study were evaluated in our Center for the Chronic Myeloproliferative Disorders and all blood samples were obtained between 5/2005 and 1/2008. Clinical parameters including cell counts, family history, chemotherapy and spleen size were extracted from the medical record generated at the time blood was collected for analysis. Splenomegaly was considered present if the spleen was palpable by physical exam and was reported as centimeters (cm) below the costal margin with a maximum of 14 cm; patients who had undergone splenectomy were also given a score of 14 cm.

Cell Processing and CD34⁺ Cell Culture

Neutrophil isolation and DNA preparation were performed as described previously(14). Peripheral blood CD34⁺ cells were isolated from Ficoll buffy coats using immunomagnetic beads (Miltenyi, Auburn, CA). CD34⁺ cells were analyzed for the expression of CD34, CD38, CD117 and CD41 using commercially available labeled antibodies. Fluorescence of at least 10,000 cells was measured on a FACS Caliber and analyzed with Cellquest and Paint-a-gate software (BD Biosciences, San Jose, CA). Similar to other published reports of the CD34⁺ cell phenotype in the MPD(15), the peripheral blood CD34⁺ cells were greater than 95% positive both for CD34 and CD38, were dimly positive for CD117, were CD41 negative, and were primarily in G₀/G₁ of the cell cycle. Purified peripheral blood CD34⁺ cells were cultured using Methocult semisolid media with growth factors (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's recommendations and incubated in a humidified environment

at 37°C for 12 days. BFU-E and CFU-GM colonies, identified by visual inspection, were plucked for isolation of genomic DNA.

JAK2^{V617F} Determination—Quantitative JAK2^{V617F} allele percentage was performed as previously described using an allele-specific, quantitative real-time PCR assay sensitive to 10% of either the wild-type or mutant JAK2 allele(14).

Statistical Analysis

We used the t-test for continuous variables and the chi-square test for categorical variables as appropriate. P-values less than or equal to 0.05 using a two-tailed test were considered significant. Statistical calculations were performed using SigmaStat (SSI, San Jose, CA).

Results

Neutrophil JAK2^{V617F} allele burden associates with both disease class and disease manifestations

Table 1 shows the epidemiologic features of the 138 JAK2^{V617F}-positive PV, 45 ET and 29 PMF patients. Marked splenomegaly, defined as greater than 8 cm below the costal margin by physical exam, was present in 17% of PV patients, and 7% required splenectomy. Of the 30% of patients on chemotherapy at the time of the study, 82% received hydroxyurea, 11% interferon and 6% anagrelide. The median disease duration at the time of analysis for the entire cohort was 7 years; 22% of the patients were studied at one year or less from their diagnosis. As observed previously, neutrophil JAK2^{V617F} allele burdens were significantly lower in ET compared to PV or PMF (14;16–18); women had lower neutrophil JAK2^{V617F} allele burdens compared to men in ET and PV, although this was only significant in PV ($p=0.01$), while women had significantly higher neutrophil allele burdens compared to men in PMF ($p=.02$) (Table 1).

We tested the association of the quantitative neutrophil JAK2^{V617F} allele burden to disease features including disease duration at the time of cell collection, platelet count, reticulocyte count spleen size and white cell count in 33 ET, 94 PV and 23 PMF patients who were not receiving chemotherapy. In ET and PMF, the neutrophil allele burden significantly correlated only with the white cell count ($R=.44$, $p=.01$ in ET and $R=.50$, $p=.02$ in PMF; Supplemental data Figure S1). In PV, the neutrophil JAK2^{V617F} allele burden inversely correlated with platelet count ($R=.31$, $p=.03$), and directly correlated with the absolute reticulocyte count ($R=.57$, $p<.001$), white cell count ($R=.64$, $p<.001$) and spleen size ($r=.42$, $p=.002$; Supplemental data Figure S1).

CD34 cell JAK2^{V617F} allele burden associates with both disease class and disease manifestations

JAK2^{V617F}-positive cells could predominate in the peripheral blood due to either an advantage in terminal differentiation of the JAK2^{V617F}-positive committed progenitor cells over residual normal committed progenitors(8;19;20), or due to the clonal expansion of JAK2^{V617F}-positive progenitor cells(21) at the progenitor level. Measuring the burden of JAK2^{V617F} at the progenitor level should distinguish between these possibilities and might prove to be more informative with regard to association with disease endpoints. CD34⁺ cells isolated from peripheral blood of MPD patients provide access to such a progenitor population, behave biologically similarly to marrow-derived MPD(22) progenitors and can be assessed for all three phenotypes, PV, PMF and ET, whereas marrow derived CD34⁺ cells are generally not available in PMF patients and many PV patients. We, therefore, assessed the relationship of the peripheral blood CD34⁺ JAK2^{V617F} allele burden to disease phenotype in 96 MPD patients. As shown in Figure 1, CD34⁺ JAK2^{V617F} cell allele burdens were significantly lower in ET

compared to PV or PMF. CD34⁺ cell JAK2^{V617F} allele percentages were significantly lower compared to neutrophil allele percentages in ET and PV, while there was no significant difference between neutrophil and CD34⁺ cell JAK2^{V617F} allele percentages in PMF.

Within PV, similar to the findings in neutrophils, the CD34⁺ cell JAK2^{V617F} allele burden inversely correlated with platelet count ($R=.34$, $p=.02$), and directly correlated with the absolute reticulocyte count ($R=.57$, $p<.001$), white cell count ($R=.70$, $p<.001$) and splenomegaly ($R=.37$, $p=.008$) (Supplemental Figure S2). In contrast to the findings in neutrophils, however, the CD34⁺ cell JAK2^{V617F} allele burden significantly increased over time ($R=.45$, $p<.001$) (Supplemental Figure S2).

CD34⁺ cell progeny recapitulated the CD34⁺ JAK2^{V617F} allele burden

In the majority of the ET and approximately half of the PV patients, the CD34⁺ cell JAK2^{V617F} allele burdens were less than 50%, suggesting the presence of JAK2^{WT} progenitor cells (Figure 1). To further define this observation, we cultured CD34⁺ cells from 6 ET, 13 PV and 6 PMF patients and determined the individual JAK2^{V617F} genotype in twenty to forty burst-forming-units-erythroid (BFU-e) from each patient.

The results for the 6 ET, 13 PV and 6 PMF patients studied in this fashion are presented in Figure 2, sorted by disease class and decreasing percentage of JAK2^{WT} clones. JAK2^{WT} clones were present in more than 50% of the colonies tested in the majority of ET and many PV patients, but this was not observed in the PMF patients. JAK2^{V617F} homozygous BFU-e clones were present primarily in PV and PMF cases (only one clone in one ET patient was homozygous) and while homozygous clones were mainly restricted to PV and PMF cases, not all PV and PMF cases were characterized by the presence of homozygous clones; a considerable proportion of PV and PMF patients had predominantly heterozygous colonies (3/13 PV, 2/6 PMF), a percentage that is identical to a recently published report (8). The presence of all three possible clones, JAK2^{WT}, JAK2^{V617F} heterozygous and JAK2^{V617F} homozygous, was a feature of the majority of PV patients (8/13 patients), while this was not observed in either the ET or PMF patients. We observed that in PV, with the exception of case 7, the presence of greater than 50% JAK2^{WT} clones was associated with the absence of splenomegaly and disease durations of less than ten years.

In the majority of ET and many of the PV patients, the neutrophil JAK2^{V617F} allele burden measured from the same blood sample from which the CD34⁺ cells were isolated was much higher than the calculated CD34⁺ cell JAK2^{V617F} allele burden derived from JAK2 genotypes of the cloned BFU-e, or from the measured CD34⁺ cell allele burden from an aliquot of CD34⁺ cells from the same cell isolation used for the BFU-e cloning experiment (Figure 2), such that the neutrophil allele burden overestimated the size of the JAK2^{V617F}-positive clone at the CD34⁺ cell level. In contrast, in PMF, the neutrophil JAK2^{V617F} allele burden better reflected the disease clone genotype because the number of residual JAK2^{WT} colonies was very low (Figure 2). Indeed, the correlations between the neutrophil and CD34 cell JAK2^{V617F} allele burdens varied between the MPD disease classes, with correlation coefficients of 0.96 in PMF 0.80 in PV and 0.67 in ET (Figure 3). The significance of differences between the three regressions was evaluated by an analysis of covariance, which found a significant difference between the regressions of PMF versus PV ($p<.00006$) and PMF versus ET ($p=.000001$), whereas there was no significant difference between that of PV versus ET.

JAK2^{V617F} Clonal Dominance at the CD34⁺ Cell Level Associates with disease class and disease features within PV

In some ET, PV and PMF subjects only JAK2^{V617F}-positive clones were present, and we defined these subjects to be JAK2^{V617F} clonally dominant at the progenitor level (Figure 2). We observed that in these particular subjects (subjects 5,6,16–19,22–25) the absolute numerical difference between the measured neutrophil and CD34⁺ cell JAK2^{V617F} allele burdens was 10 or less (Figure 2). We therefore arbitrarily defined an absolute difference between the quantitative neutrophil and CD34⁺ JAK2^{V617F} allele burdens of 10 or less as an indicator of “clonal dominance” at the progenitor level, and tested the associations of clonal dominance between disease classes and within PV disease features in 17 ET, 64 PV and 15 PMF patients. The prevalence of clonal dominance was significantly different between the three disease classes, present in 4/17 (24%) ET, 36/64 (56%) PV and 14/15 (93%) PMF patients ($p < 0.05$). PV patients who were clonally nondominant at the progenitor level had significantly shorter disease durations, lower white cell counts, and smaller spleens compared to PV patients who were clonally dominant at the progenitor level (Table 2). Importantly, the neutrophil allele burdens in the clonally dominant group were not significantly different than the nondominant group (mean 68.1 vs 62.5%; $p = 0.29$) and therefore, could not reliably distinguish these two groups (Table 2).

Discussion

The World Health Organization (WHO) currently classifies PV, PMF and ET together with chronic myelogenous leukemia, chronic eosinophilic leukemia, chronic neutrophilic leukemia, the hypereosinophilic syndrome and unclassifiable chronic myeloproliferative disease under the rubric of the chronic myeloproliferative disorders(23). However, PV, PMF and ET have more in common phenotypically with each other than with these other disorders and recently this association was confirmed by the discovery of the JAK2^{V617F} mutation, the expression of which is largely confined to PV, PMF and ET(24;25).

If one assumes that JAK2^{V617F} is central to MPD pathogenesis, the observation that different disease classes associate with the identical mutation requires explanation. The issues that may be important to consider with respect to this are the gene dosage within the disease clone, and the extent of clonal dominance of that clone over the normal polyclonal hematopoiesis. In contrast to CML, in which the BCR/ABL-positive clone is uniformly dominant at the neutrophil level, in ET, PV and PMF, clonal dominance at the circulating blood cell level is not always observed(26–28). To examine these issues, we measured the percentage of JAK2^{V617F} alleles in PV, PMF and ET neutrophils, CD34⁺ cells, and cultured erythroid colonies using a quantitative, allele-specific assay. Our study population consisted of a large, clinically well-defined group of PV, PMF and ET patients followed for up to 30 years.

Our observation in PV and ET that the neutrophil JAK2^{V617F} allele burden was often much higher than the CD34⁺ cell allele burden indicated that JAK2^{V617F} is advantageous to the terminal stages of hematopoietic differentiation, a finding recently reported by others(8). Thus, in PV and ET, the neutrophil JAK2^{V617F} allele burden can over-estimate the burden at the progenitor cell level, which can be variable in both ET and PV (Figure 1–Figure 3). Moreover, JAK2^{V617F} has been identified only in platelets in some ET patients, and at higher levels in some of these patients compared to their neutrophils, again suggesting that JAK2^{V617F} may also be differentially advantageous to specific myeloid lineages at their terminal maturation stage(29–31).

In PV, an analysis of the CD34⁺ cell JAK2^{V617F} allele burden revealed a stronger correlation to disease endpoints such as splenomegaly, white cell count and platelet count than the neutrophil JAK2^{V617F} allele burden, and also revealed that the CD34⁺ cell JAK2^{V617F} allele

burden increased over time in PV, as was previously demonstrated using G-6PD isoenzyme analysis(7). The fact that CD34⁺ JAK2^{V617F} allele burden more strongly correlated with some disease features than the neutrophil JAK2^{V617F} allele burden in PV indicates that the expansion or dominance of JAK2^{V617F}-positive over JAK2^{V617F}-negative cells at the CD34⁺ cell level is an important biologic factor in the evolution of this disorders, a finding also observed in murine models of JAK2^{V617F}-positive myeloproliferative disease(32). In contrast, in PMF, the JAK2^{V617F}-bearing clone was dominant at the progenitor level independent of disease duration. Thus, the JAK2^{V617F}-positive clone behaves differently biologically in de novo PMF compared to ET or PV, since it exhibits clonal dominance and expansion at disease onset, a feature reflected in the increase in circulating CD34⁺ cells in the early stages of PMF(15).

Our data also speak to the assessment of JAK2^{V617F}-targeted therapy in the MPD. Studies in JAK2 knockout mice have indicated that JAK2 is not obligatory for primitive stem cell proliferation and lineage-specific commitment but rather, only for the survival of committed erythroid, myeloid and megakaryocytic cells(33). Thus, JAK2^{V617F} enhanced the terminal amplification and development of committed cells in the erythroid lineage (3;34), granulocyte lineage(35) and platelets(30;31), and made committed myeloid progenitor cells hypersensitive to hydroxyurea(36). However, neither hydroxyurea(37;38) nor other forms of chemotherapy (39–41) prevent MPD progression. Therefore, therapies directed against the JAK2^{V617F}-bearing clones will have to be active at the primitive progenitor level in order to do more than suppress disease activity, and analysis of therapeutic efficacy will require assessment for residual disease at the primitive progenitor cell level.

We conclude that JAK2^{V617F} clones behave biologically differently at the stem cell level in ET, PV and PMF, and that the degree of expansion or domination at the CD34⁺ progenitor cell level is an important modifier of the MPD disease phenotype. However, given that these diseases are indolent, often requiring decades to fully declare their phenotype in an individual patient, genetic, gender and environmental factors that influence the behavior of the disease clone at the stem cell level will also be important to identify as they may provide clinically relevant diagnostic and prognostic information(42–44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Quantitative JAK2^{V617F} allele burden in neutrophils and CD34⁺ cells obtained from the same blood sample in 17 ET, 64 PV and 15 PMF patients. Medians are indicated by bars, coincident points are overlapping.

Figure 2.

Relationship of BFUe JAK2^{V617F} genotypes to MPD subtype, disease duration, sex, splenomegaly, and measured CD34⁺ cell and neutrophil JAK2^{V617F} allele burdens in 25 patients. On average, 27 colonies were plucked from each cloning experiment (range 20–40). Clear bars indicate a JAK2^{WT} genotype, striped a heterozygous JAK2^{V617F} genotype and black a homozygous JAK2^{V617F} genotype. Asterisks above case number (top) indicate antecedent history of ET in all cases with the exception of 25, where there was an antecedent history of PV.

Figure 3. Relationship of Neutrophil JAK2^{V617F} allele burden to CD34 cell JAK2^{V617F} allele burden in 17 ET, 64 PV and 15 PMF patients. Dotted line indicates the regression line for an R=1.0 for reference.

Table 1
Clinical and epidemiologic features of the JAK2^{V617F}-positive MPD cohort.

	ET	PV	PMF
N	45	138	29
% female	66	64	39
% antecedent MPD [↓]	NA	20	27
Median disease duration, years (range)	4 (0–21) [§]	8 (0–28)	7 (0–20)
% with family MPD history	22	6	6
% with palpable splenomegaly	6	44	100
% receiving chemotherapy	27	31	21
Mean age at diagnosis			
Females (range)	53 (27–84)	51 (15–88) [*]	57 (27–70)
Males (range)	52 (21–78)	57 (30–74)	58 (53–78)
Mean neutrophil JAK2 ^{V617F} %			
Total	38 [#]	68	70
Females (range)	36 (13–61)	68 (29–100) [†]	82 (45–100) [‡]
Males (range)	41 (17–63)	74 (32–100)	62 (43–100)

[↓] 20% of both PV and PMF patients had a prior history of ET, while 7% of the PMF patients had a prior history of PV.

[§] Median disease duration ET vs PV, p= 0.006.

^{*} Age at PV diagnosis, female versus male, p = .04

[#] Allele burden PV vs ET, p = <.001

[‡] female vs male PMF, p=.02

Table 2

Comparison of clinical features of clonally dominant versus nondominant PV patients in 50 patients not receiving chemotherapy.

	Clonally dominant PV	Clonally nondominant PV	P value
Number	26	24	
Disease duration (years)	8.1 (0–25)	3.0 (0–10)	0.007
Platelet count (K/cu mm)	526 (38–1239)	663 (291–1269)	0.12
Reticulocyte count (K/cu mm)	102.3 (45.5–232.2)	94.6 (62.2–138.2)	0.43
Spleen size (centimeters below the costal margin)	4.3 (0–14)	1.3 (0–14)	0.05
White cell count (K/cu mm)	20.4 (4.5–47.7)	13.4 (5.8–37.2)	0.01
Neutrophil allele burden (%)	68.1 (40–100)	62.5 (35–100)	0.29
CD34 cell allele burden (%)	70.5 (35–100)	36.3 (11–87)	<.001

All values are expressed as means and ranges (parentheses).