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ORIGINAL ARTICLE

The implication of identifying *JAK2*^{V617F} in myeloproliferative neoplasms and myelodysplastic syndromes with bone marrow fibrosis

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Abstract The myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) occasionally demonstrate overlapping morphological features including hypercellularity, mild/nonspecific dysplastic changes and variable bone marrow fibrosis. Thus, when the associated bone marrow fibrosis results in a suboptimal specimen for morphological evaluation, the descriptive diagnosis "fibrotic marrow with features indeterminate for MDS versus MPN" is often applied. The *JAK2*^{V617F} mutation was recently shown to be frequently identified in MPN, but it is rarely present in other myeloid disorders. However, the diagnostic utility of *JAK2*^{V617F} screening in hypercellular bone marrow specimens with fibrosis has not been previously investigated. Using a real-time polymerase chain reaction melting-curve assay capable of detecting *JAK2*^{V617F} in archived fixed materials, we retrospectively studied *JAK2*^{V617F} in 45 cases with fibrotic hypercellular bone marrow at initial presentation, including 19 cases initially described as "with features indeterminate for MDS versus MPN". These 19 cases were reclassified into more specific categories of MDS (n=14) or MPN (n=5) based on the availability of subsequent clinical data and/or bone marrow examinations. The *JAK2*^{V617F} allele was identified in 17 out of 18 BCR/ABL gene-negative MPN cases with marrow fibrosis, whereas only wild-type alleles were identified in the remaining non-MPN cases. Importantly, *JAK2*^{V617F} alleles were seen in all five cases of "with features indeterminate for MDS versus MPN" at initial presentation that were later determined to be MPN, but they were absent in the 14 cases later determined to be MDS. Our results suggest that *JAK2*^{V617F} allele evaluation can be a useful ancillary test for discriminating MDS from MPN in specimens with bone marrow fibrosis.

Keywords Myeloproliferative neoplasm · Myelodysplastic syndrome · Bone marrow fibrosis · JAK2^{V617F}

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Introduction

The myeloproliferative neoplasms (MPN, also known as chronic myeloproliferative disorders or MPD) and myelodysplastic syndromes (MDS) are usually distinguished by

their clinical presentation, laboratory parameters, and morphological appearance. However, they occasionally demonstrate overlapping features including the coexisting presence of mild hypercellularity, mild/nonspecific dysplasia, and variable bone marrow fibrosis [1, 2]. Cases with fibrosis may be problematic due to the difficulties associated with obtaining an adequate aspirate smear specimen for optimal microscopic examination, particularly when complete clinical information and/or a peripheral blood smear is not available. In these cases, descriptive diagnoses, such as "with features indeterminate for MDS versus MPN", are usually given, and follow-up biopsies may be necessary for rendering specific diagnoses. New molecular markers that better discriminate these morphologically similar but biologically distinct entities could significantly improve clinical management and facilitate research studies by providing accurate diagnoses at the time of initial presentation [1–3].

A specific mutation in the *Janus kinase 2* gene ($JAK2^{V617F}$) was recently shown to be frequently and preferentially identified in the bone marrow and peripheral blood cells of MPN patients [1, 2, 4–20]. The $JAK2^{V617F}$ allele has been detected in the vast majority of polycythemia vera (PV) cases, in the majority of essential thrombocytosis (ET) and primary myelofibrosis (PMF) cases, and in many acute leukemias representing transformation from preexisting MPN. However, $JAK2^{V617F}$ is rarely identified in healthy controls or patients with other myeloid disorders. Thus, $JAK2^{V617F}$ has general diagnostic value for MPN, but it cannot be used to differentiate between PV, ET, or PMF [1, 2, 4–19].

The diagnostic utility of $JAK2^{V617F}$ mutation screening in hypercellular bone marrow specimens with fibrosis has not been previously investigated. We retrospectively evaluated the JAK2 genotype of 45 fibrotic bone marrow specimens, including 19 cases that were originally diagnosed as "with features indeterminate for MDS versus MPN" using our assay that reliably detects $JAK2^{V617F}$ in archived and paraffin-embedded materials [20, 21]. Our results demonstrated that the presence or absence of $JAK2^{V617F}$ may have diagnostic implications for these cases.

Materials and methods

Patient samples

Archival pathology and hematology records at our respective institutions were retrospectively reviewed to identify patients with a mildly to markedly fibrotic bone marrow biopsy at initial marrow evaluation. Using reticulin and collagen stains, fibrosis was graded on a scale of 0 to 3 as previously described [22]. For PMF cases, grade 1 was considered the early/prefibrotic stage (also termed cellular phase) and grades 2–3 was considered the fibrotic stage [22, 23]. The cohort was limited to nonchronic myelogenous leukemia (*BCR/ABL* fusion gene negative) patients with adequate history and follow-up for clinicopathologic correlation. Forty-five specimens were identified as follows: 19 cases initially assigned to "with features indeterminate for MDS versus MPN" because of fibrosis-associated inadequate aspirate and/or lack of complete clinical information, 11 cases with a confirmed MPN (two PMF prefibrotic stage, four PMF fibrotic stage, three PV at spent phase, two ET, and four PMF), two cases with acute myelogenous leukemia (AML) transformed from a preexisting PMF, two cases with chronic myelomonocytic leukemia, and 11 cases with other neoplastic myeloid disorders (Table 1). None of these cases were tested for JAK2 mutation at the time of initial evaluation. This study was approved by the Institutional Review Board of all participating institutions, and samples were obtained in accordance with institutional policies.

DNA extraction and real-time PCR melting curve analysis for JAK2 genotype

For each case, DNA was extracted from stained or unstained peripheral blood smears, stained or unstained bone marrow aspirate smears, or formalin-fixed paraffin-embedded bone marrow clot sections using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) as previously described [20, 21]. There was a relatively even distribution of each specimen type within each disease category (Table 1). Real-time polymerase chain reaction (PCR) melting curve analysis for the *JAK2* wild-type and V617F mutant allele was performed on a LightCycler platform (Roche Applied Diagnostics, Indianapolis, IN, USA) using primers and probes as previously described [20, 21]. Briefly, PCR primers were designed to flank codon 617 of the JAK2 gene, including forward primer JAKLCFP 5′-AAg CAg CAA gTA TgA TgA gCA A-3′ and reverse primer JAKLCRP 5′-AgC TgT gAT CCT gAA ACT gAA-3′. FRET probes were designed with the 5′ probe overlapping the mutated codon and the 3′ probe annealing immediately downstream, including LCRD 5′-640-CAg ACA CAT ACT CCA TAA TTT-3′ and LCFN 5′-gTA gTT TTA CTT ACT CTC gTC TC-FITC-3′. Real-time PCR was performed on each specimen using 5.0 μL of purified DNA extract in a total reaction volume of 20 μL that included 4 μL of FastStart DNA MasterPLUS SYBR Green I 5× reaction master mix (Roche Applied Science), 2.0 μL JAK2LCFP (final concentration 0.5 μM), 2.0 μL JAK2LCRP (final concentration 0.5 μM), 1.0 μL LCFN (final concentration 0.5 μM), 1.0 μL LCRD (final concentration 0.5 μM), and 5.0 μL nuclease-free water. The PCR cycle parameters were one initial denaturing step of 95°C for 10 min and 55 cycles consisting of 95°C for 10 s, 60°C for 60 s, and 75°C for 10 s. The DNA melting curve analysis was performed by denaturing at 95°C for 10 s, annealing at

Table 1 Results of *JAK2*^{V617F} genotyping in fibrotic bone marrow specimens

Case no.	Specimen type used for molecular testing	Initial diagnosis assigned based on first diagnostic evaluation	Final diagnosis following subsequent clinical–morphological evaluations	Fibrosis (grades 0–3)	JAK2 genotype
1	Aspirate	"Features indeterminate for MDS versus MPN"	Prefibrotic CIMF	1	Mutant
2	Clot section	"Features indeterminate for MDS versus MPN"	Prefibrotic CIMF	1	Mutant
3	Peripheral blood	"Features indeterminate for MDS versus MPN"	Prefibrotic CIMF	1	Mutant
4	Clot section	"Features indeterminate for MDS versus MPN"	CIMF, fibrotic stage	3	Mutant
5	Aspirate	"Features indeterminate for MDS versus MPN"	CIMF, fibrotic stage	3	Mutant
6	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
7	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	3	Wild type
8	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	3	Wild type
9	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
10	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
11	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	3	Wild type
12	Aspirate	"Features indeterminate for MDS versus MPN"	MDS-F	3	Wild type
13	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	3	Wild type
14	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	1	Wild type
15	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
16	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
17	Peripheral blood	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
18	Clot section	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
19	Clot section	"Features indeterminate for MDS versus MPN"	MDS-F	2	Wild type
20	Aspirate	Prefibrotic CIMF	prefibrotic CIMF	1	Mutant
21	Clot section	Prefibrotic CIMF	prefibrotic CIMF	1	Mutant
22	Clot section	CIMF, fibrotic stage	CIMF, fibrotic stage	3	Mutant
23	Clot section	CIMF, fibrotic stage	CIMF, fibrotic stage	3	Mutant
24	Clot section	CIMF, fibrotic stage	CIMF, fibrotic stage	3	Mutant
25	Peripheral blood	CIMF, fibrotic stage	CIMF, fibrotic stage	3	Mutant
26	Aspirate	AML transformed from CIMF	AML transformed from CIMF	3	Mutant
27	Aspirate	AML transformed from CIMF	AML transformed from CIMF	2	Mutant
28	Aspirate	ET with mild fibrosis	ET with mild fibrosis	1	Mutant
29	Aspirate	ET with mild fibrosis	ET with mild fibrosis	1	Wild type
30	Aspirate	PV, PPMM	PV/PPMM	3	Mutant
31	Peripheral blood	PV, PPMM	PV/PPMM	3	Mutant
32	Peripheral blood	PV, PPMM	PV/PPMM	3	Mutant
33	Aspirate	ALL with marked fibrosis	ALL with marked fibrosis	3	Wild type
34	Aspirate	ALL with marked fibrosis	ALL with marked fibrosis	3	Wild type
35	Aspirate	ALL with marked fibrosis	ALL with marked fibrosis	3	Wild type
36	Aspirate	AML with mild fibrosis	AML with mild fibrosis	1	Wild type
37	Aspirate	AML with moderate fibrosis	AML with moderate fibrosis	2	Wild type
38	Aspirate	AML with moderate fibrosis	AML with moderate fibrosis	2	Wild type
39	Aspirate	AML with moderate fibrosis	AML with moderate fibrosis	2	Wild type
40	Aspirate	CLL with mild fibrosis	CLL with mild fibrosis	1	Wild type
41	Aspirate	CMML with t(5;12), eosinophilia & fibrosis	CMML with t(5;12), eosinophilia & fibrosis	2	Wild type
42	Peripheral blood	CMML with moderate fibrosis	CMML with secondary fibrosis	2	Wild type
43	Aspirate	LGL leukemia with moderate fibrosis	LGL leukemia with secondary fibrosis	2	Wild type
44	Aspirate	Mastocytosis with fibrosis	Mastocytosis with fibrosis	3	Wild type
45	Aspirate	TCC with secondary fibrosis	TCC with secondary fibrosis	3	Wild type

Cases 1–19 were originally descriptively diagnosed as "with features indeterminate for MDS versus MPN" due to insufficient clinicopathological evidence for a specific diagnosis, but each was later reclassified, independent of *JAK2* allele status, as either CIMF or MDS with myelofibrosis (MDS-F) based on the evaluation of subsequent bone marrow specimens and correlation with clinical disease progression. *JAK2* genotype is reported as wild-type or mutant, and fibrosis is graded on a scale of 0–3.

MDS Myelodysplastic syndromes, *MPN* myeloproliferative neoplasms, *CIMF* chronic idiopathic myelofibrosis, *AML* acute myeloid leukemia, *ET* essential thrombocythemia, *PV* polycythemia vera, *PPMM* post-polycythemic myeloid metaplasia, *ALL* acute lymphoblastic leukemia, *CLL* chronic lymphocytic leukemia, *CMML* chronic myelomonocytic leukemia, *LGL* large granular lymphocyte, *TCC* transitional cell carcinoma

29°C for 60 s, and melting by a transition rate of 0.20 C/s to 70 C. Melting curves were visually analyzed, and the melting temperature (T_m) of each sample was electronically recorded. Homozygous mutant ($JAK2^{V617F}$/$JAK2^{V617F}$) human erythroleukemia (HEL) and homozygous wild-type (*JAK2*/*JAK2*) multiple myeloma (RPMI8226) cell lines were used as positive and negative controls, respectively (Fig. 1).

Results

Medical records and bone marrow specimens obtained at subsequent evaluations were reviewed for all the patients to render final diagnoses using World Health Organization criteria to the fullest extent possible [1, 23–25]. Of note, the 19 cases initially designated as "with features indeterminate for MDS versus MPN" were ultimately reclassified as either PMF (n=5) or MDS with myelofibrosis (MDS-F, n=14; Table 1) based on repeat/follow-up biopsies and/or clinical progression. None of these cases fulfilled the criteria for the diagnosis of MDS/MPN according to WHO classification. The blast count in the cases finally diagnosed as MDS-F could not be accurately evaluated due to inadequate marrow smear, and thus, a definite subtype of MDS could not be assigned accurately. However, the CD34 stain showed an increase of blasts in the majority of these cases, suggesting they were high-grade MDS (refractory anemia with excess of blasts). All diagnoses were assigned without the prior knowledge of *JAK2* mutation testing results.

The *JAK2* gene was successfully amplified by our real-time PCR melting curve assay in all 45 cases (examples shown in Fig. 1). The mutant allele was detected in 17 out of 18 (94%) patients with a MPN (according to the final diagnosis in Table 1), including five out of five cases of prefibrotic PMF, six out of six cases of PMF in the fibrotic stage, two out of two cases of AML transformed from preexisting PMF, one out of two cases of ET with fibrosis, and three out of three cases of PV with fibrosis (also termed post-polycythemic myeloid metaplasia, PPMM; Table 1). Only the wild-type allele was detected in 27 out of 27 (100%) patients with other neoplastic myeloid disorders associated with fibrosis, including 14 out of 14 cases of MDS-F, three out of three cases of ALL with marked fibrosis, four out of four cases of AML with mild to moderate fibrosis, two out of two cases of CMML with moderate fibrosis, one out of one case of CLL with mild fibrosis, one out of one case of large granular lymphocytic leukemia with moderate fibrosis, one out of one case of mastocytosis with marked fibrosis, and one out of one case of metastatic transitional cell carcinoma with marked fibrosis (Table 1).

Of note, 19 cases (Table 1, cases 1–19) were originally descriptively diagnosed as "with features indeterminate for MDS versus MPN" due to insufficient clinicopathological evidence for definitive categorization. Figure 2 illustrates the significant overlapping features of MDS and MPN in

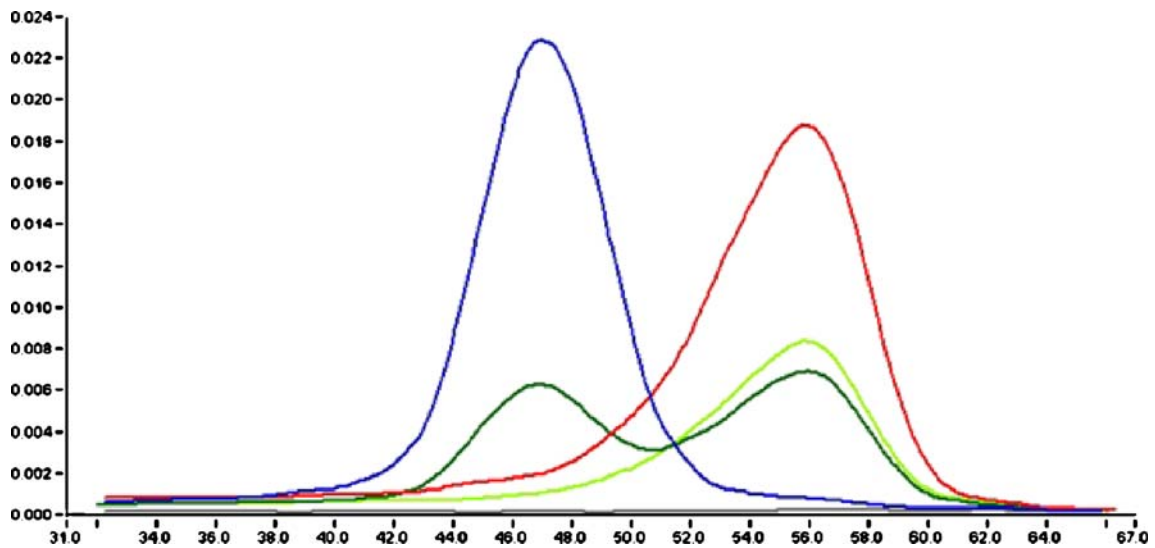

Fig. 1 Representative *JAK2* real-time PCR melting curves. Melting curves are drawn with $-dF/dT$ on the y-axis and melting temperature (T_M,°C) on the x-axis. The homozygous wild-type (*JAK2*/*JAK2*) multiple myeloma control cell line (RPMI 8226) is shown in *red* with the melting curve peak at approximately 56°C (T_M=56°C), and the homozygous mutant ($JAK2^{V617F}$/$JAK2^{V617F}$) human erythroleukemia control cell line (HEL) is shown in *blue* with the melting curve peak at approximately 47°C (T_M=47°C). A representative case of MDS-F (*JAK2* wild type) is shown in yellow with a single T_M equivalent to the wild-type *JAK2* allele. A representative case of prefibrotic PMF (heterozygous $JAK2/JAK2^{V617F}$) is shown in *green* with two T_M; one peak corresponds to the *JAK2* wild-type allele and the other corresponds to the $JAK2^{V617F}$ mutant allele

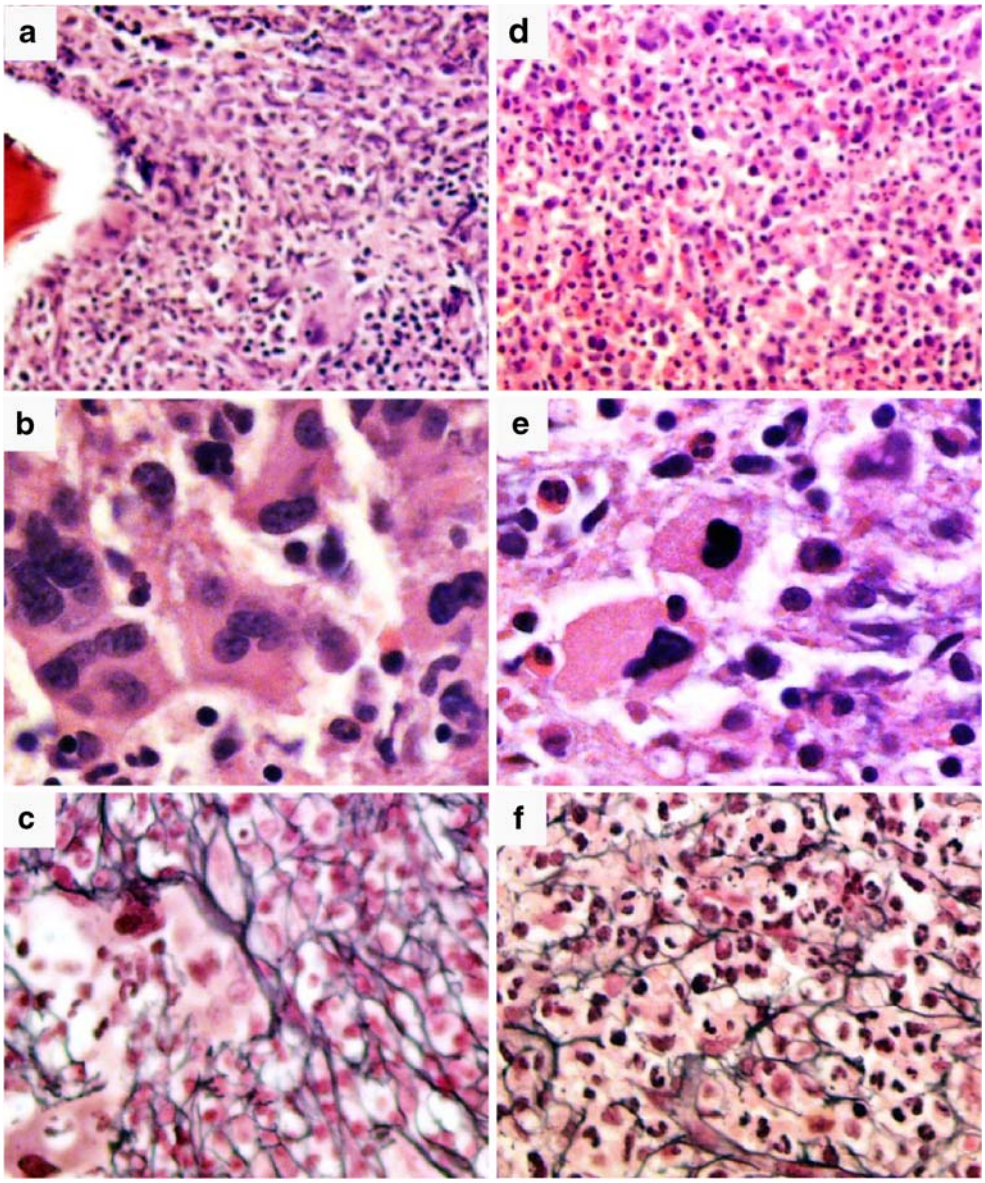

Fig. 2 Microscopic evaluation of fibrotic bone marrow specimens. Representative micrographs of two cases with fibrotic marrow and "features indeterminate for MDS versus MPN" at the time of initial clinical presentation, that were later reclassified as PMF (**a**–**c**) and MDS-F (**d**–**f**). Low power (**a** and **d**, respectively), high power (**b** and **e**, respectively), and reticulin stains (**c** and **f**, respectively) are shown. Both cases demonstrate overlapping morphological features including hypercellularity, mild dysplasia and fibrosis, making a definitive diagnosis difficult. **b** demonstrates the increased bizarre megakaryocytes often observed in PMF, and **e** demonstrates the mononucleated megakaryocytes often seen in MDS. However, the morphologic evaluation alone as shown at initial presentation is not sufficient to render a specific diagnosis without follow-up biopsies and additional clinical findings

two representative cases at initial presentation. One (panel A to C) was later determined to be prefibrotic PMF, and the other was reclassified as MDS-F with additional data obtained from follow-up biopsies and/or clinical findings. Importantly, *JAK2*^{V617F} mutant alleles were detected in all cases reclassified as PMF (Table 1, cases 1–5), whereas only *JAK2* wild-type alleles were detected in all cases reclassified as MDS-F (Table 1, cases 6–19).

Discussion

Our results suggest that *JAK2*^{V617F} mutation screening is an important ancillary test for distinguishing between MDS and other *BCR-ABL* negative MPN with marrow fibrosis. Distinction between MPN and MDS is important for the appropriate clinical management of hematology patients. However, some cases may occasionally demonstrate overlapping morphological, laboratory, and clinical features that result in considerable diagnostic difficulty, particularly when the marrow aspirate is not optimal for morphologic evaluation due to the associated marrow fibrosis and when clinical data is limited or peripheral blood smears are unavailable for review. Our results demonstrated that *JAK2*^{V617F} was detected in nearly all cases of *BCR-ABL* negative MPN with fibrosis (Table 1). Only one case of *JAK2*^{V617F}-negative ET was identified. This particular patient may carry one of the other more rare *JAK2* or MPL mutations not detected by our real-time PCR assay [26, 27]. In comparison, only wild-type alleles were detected in each case diagnosed as a non-MPN myeloid disorder with marrow fibrosis (Table 1). Other reports have

recently demonstrated the occurrence of *JAK2*^{V617F} in MDS/MPN cases, including chronic myelomonocytic leukemia and atypical chronic myelogenous leukemia [28–30]. However, compared to MPN, a lower frequency of JAK2 mutations was identified. Taken together, analysis of the JAK2 mutation, in difficult-to-classify cases, will help to clarify the borderline between MDS (lacking JAK2 mutation) on one hand, and atypical MPN and MDS/MPN on the other hand. It would have been interesting to also include cases of autoimmune-associated marrow fibrosis, another setting that may mimic MPN or MDS associated with marrow fibrosis. However, no cases were found in our patient databases. Additionally, in our series, we found that acute leukemia arising in PMF retain the *JAK2*^{V617F}-positive genotype, whereas no *JAK2* mutations were found in de novo AML with fibrosis. Since AML transformed from a preexisting MPN has a poorer overall prognosis, *JAK2* genotyping may also have value in stratifying risk groups or predicting therapeutic responses in acute myeloid leukemia patients [31, 32].

Our observations suggest that *JAK2*^{V617F} testing is particularly helpful for cases with marrow fibrosis that are difficult to classify into MDS or MPN at the initial presentation [26]. Among the 19 specimens initially described as "with features indeterminate for MDS versus MPN", *JAK2*^{V617F} mutant alleles were detected in each case that was ultimately reclassified as PMF (Table 1, cases 1–5), whereas only wild-type alleles were detected in the cases eventually reclassified as MDS-F (Table 1, cases 6–19). Although each patient was reclassified based on the evaluation of subsequent morphological and clinical information, *JAK2*^{V617F} genotyping could have substantially aided diagnosis at the time of initial presentation since the retrospective *JAK2*^{V617F} studies were concordant with the final disease phenotype. The JAK genotyping results, together with other clinical–morphological data and follow-up information, will be important for rendering a definitive diagnosis as early as possible during the disease course. This will emerge as an increasingly important capability when specific treatments targeting the *JAK2* signaling pathway become available.

JAK2^{V617F} is not observed in well-defined MDS cases with marrow fibrosis in our cohort. This suggests that this mutation must play a minimal role, if any, in the fibrosis that occasionally occurs in MDS. However, it remains controversial whether *JAK2*^{V617F} is associated with MDS with fibrosis. Initially, a report by Ohyashiki et al., described the presence of *JAK2*^{V617F} in two of six MDS cases with fibrosis, but it was absent in multiple cases of AML, lymphoma, chronic myeloid leukemia with fibrosis, and MDS without fibrosis [33]. However, the results of our cohort and another study by Kremer et al. do not support that hypothesis [34]. In agreement with our findings, they also reported that *JAK2*^{V617F} is exceedingly rare in bona fide MDS or de novo AML, regardless of the presence or absence of fibrosis [34].

The optimal assays used to detect *JAK2*^{V617F} in the setting of marrow fibrosis should be highly sensitive and capable of using formalin-fixed materials as described here. Of note, although the markedly hemodiluted marrow smears and clot sections associated with bone marrow fibrosis are suboptimal for morphological evaluation, they contained a sufficient number of clonal hematopoietic cells for successful DNA extraction and molecular testing. In our laboratory, this real-time PCR melting curve assay was previously shown to have an analytical sensitivity of 5% (i.e., capable of detecting 5 *JAK2*^{V617F}-positive cells per 100 total cells) [20, 21]. This sensitivity may at least partially attribute to the higher percentages of PMF patients carrying *JAK2*^{V617F} in our cohort than reported in the literature [4–19]. Other explanations would include biased sampling in our cohort and different types of samples tested between our study (predominantly archived marrow samples) and other studies (predominantly using fresh blood samples). Additionally, the possibility of a case selection bias for *JAK2*^{V617F} screening in the cited studies cannot be excluded. Furthermore, at times, when additional marrow aspirate slides are not available, the ability to test for this mutation using formalin-fixed clot sections will eliminate the need for subsequent phlebotomies that can significantly prolong turnaround time.

In summary, our results demonstrate that *JAK*^{V617F} is very frequently identified in fibrotic bone marrow specimens associated with *BCR/ABL* negative MPN, but it is not observed in MDS with marrow fibrosis. These findings indicate that the evaluation of *JAK2* mutation status is an important tool that may aid the diagnosis of patients with marrow fibrosis.

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