

## Two routes to leukemic transformation after a *JAK2* mutation–positive myeloproliferative neoplasm

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**Acute myeloid leukemia (AML) may follow a *JAK2*-positive myeloproliferative neoplasm (MPN), although the mechanisms of disease evolution, often involving loss of mutant *JAK2*, remain obscure. We studied 16 patients with *JAK2*-mutant (7 of 16) or *JAK2* wild-type (9 of 16) AML after a *JAK2*-mutant MPN. Primary myelofibrosis or myelofibrotic transformation preceded all 7 *JAK2*-mutant but only 1 of 9 *JAK2* wild-type AMLs ( $P = .001$ ), implying that *JAK2*-mutant AML is preceded by**

**mutation(s) that give rise to a “myelofibrosis” phenotype. Loss of the *JAK2* mutation by mitotic recombination, gene conversion, or deletion was excluded in all wild-type AMLs. A search for additional mutations identified alterations of *RUNX1*, *WT1*, *TP53*, *CBL*, *NRAS*, and *TET2*, without significant differences between *JAK2*-mutant and wild-type leukemias. In 4 patients, mutations in *TP53*, *CBL*, or *TET2* were present in *JAK2* wild-type leukemic blasts but absent from the *JAK2*-mutant**

**MPN. By contrast in a chronic-phase patient, clones harboring mutations in *JAK2* or *MPL* represented the progeny of a shared *TET2*-mutant ancestral clone. These results indicate that different pathogenetic mechanisms underlie transformation to *JAK2* wild-type and *JAK2*-mutant AML, show that *TET2* mutations may be present in a clone distinct from that harboring a *JAK2* mutation, and emphasize the clonal heterogeneity of the MPNs. (*Blood*. 2010;115(14):2891-2900)**

### Introduction

The myeloproliferative neoplasms (MPNs) comprise a group of clonal stem cell disorders associated with a high prevalence of mutations in *JAK2*, overproduction of mature blood elements, and variable rates of transformation to acute myeloid leukemia (AML).<sup>1,2</sup> AML transformation is seen in 2% to 5% of patients with essential thrombocythemia (ET) or polycythemia vera (PV)<sup>3,4</sup> and 15% to 30% of those with primary myelofibrosis (PMF).<sup>5</sup> Although rates of leukemic transformation are increased after genotoxic therapy, AML also occurs in treatment-naïve patients,<sup>4</sup> indicating that leukemic transformation is part of the natural history of these disorders. Of note, recent studies have shown a role for the *JAK2* V617F mutation in promoting the generation of clones bearing additional genetic damage, suggesting that disease evolution may be driven in part by expression of mutant *JAK2*.<sup>6,7</sup>

In striking contrast to chronic myeloid leukemia, in which the blastic phase of disease invariably harbors a *BCR-ABL1* fusion, AML transformation after a *JAK2* V617F-positive MPN commonly lacks the *JAK2* mutation. The first hint of this phenomenon came from the observation that the *JAK2* V617F mutation was less common in patients with AML transformation compared with patients with PV or PMF.<sup>8</sup> Loss of the *JAK2* mutation at leukemic transformation was subsequently shown by analysis of paired MPN and AML samples.<sup>9,10</sup> There are several models to explain the development of *JAK2* wild-type leukemia. One potential explanation would be loss of the mutant *JAK2* allele as a consequence of

mitotic recombination. This was not detected in 3 patients transforming to *JAK2* wild-type AML,<sup>9,10</sup> but, given the small number of patients analyzed, it remains possible that mitotic recombination accounts for a proportion of such cases. Moreover, the previous reports were unable to exclude gene conversion or intrachromosomal deletion. A second model invokes the concept of a “pre-*JAK2*” phase of disease. This idea is supported by circumstantial evidence, including the familial predisposition to MPN,<sup>11,12</sup> clonality studies in female patients with MPN,<sup>13,14</sup> and growth of V617F-negative endogenous erythroid colonies from V617F-positive patients.<sup>15,16</sup> More recently direct evidence has been reported that the *JAK2* mutation may be preceded by a 20q deletion<sup>17</sup> or a *TET2* mutation<sup>18</sup> in some patients, but the frequency of this occurrence remains unclear. A third model that could explain the development of *JAK2* wild-type AML would be the coexistence of independent *JAK2*-mutant and *JAK2* wild-type clonal expansions in the same patient. This phenomenon has been documented recently, and, in 2 patients informative for X-linked polymorphic loci, the 2 clones were shown to have arisen independently.<sup>19</sup>

At the time of AML transformation, blood and bone marrow samples contain cells representing the leukemic process as well as others derived from the preceding MPN. Molecular analyses therefore require careful purification of the leukemic blasts and/or analysis of clonally derived progenitor colonies. We have used these approaches to undertake a detailed study of 16 patients with

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AML after a *JAK2*-mutant MPN. Our results show that *JAK2*-mutant AML, but not *JAK2* wild-type AML, usually arises from a preceding myelofibrotic phase. Moreover, although *TET2* mutations precede *JAK2* mutations in some patients, in others the 2 mutations are present in distinct clones.

## Methods

### Patients and samples

Comprehensive clinical information and samples of blood or bone marrow were obtained from patients with AML after a *JAK2*-mutant MPN. Patients were diagnosed with ET, PV, PMF, or refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T) according to standard criteria.<sup>20</sup> Diagnostic criteria for myelofibrotic transformation of ET/PV were as previously described<sup>21</sup> and required at least grade 3 reticulin fibrosis in a bone marrow biopsy together with at least 2 of the following: increase in spleen size of at least 3 cm, unexplained decrease in hemoglobin by at least 20 g/L, immature myeloid or erythroid cells in the blood smear, tear-drop poikilocytes in the blood smear, or B symptoms (night sweats, bone pain, or weight loss of more than 10% in 6 months). Diagnosis of AML transformation required the presence of at least 20% blasts in the blood or bone marrow.<sup>20</sup>

Samples of leukemic blasts and constitutional material (derived from buccal swabs or T cells) were available from all 16 patients. Samples from the MPN phase of disease (Table 1) and/or individual progenitor colonies (Table 3) were also available from a proportion of patients. Leukemic blasts were purified when necessary by density centrifugation (Lymphoprep; Axis-Shield), collection of mononuclear cells, and magnetic isolation of CD34<sup>+</sup> cells (magnetic-activated cell sorting; Miltenyi Biotec). Constitutional material representative of germline DNA was obtained from buccal swabs (Epicentre), or magnetically isolated CD2<sup>+</sup> T cells (Dynabeads; DYNAL AB). Granulocytes were purified by density centrifugation. Leukemic blasts and granulocytes were at least 90% pure by morphologic criteria. T-cell purity was also 90% or greater, as assessed by visualizing the proportion of cells directly bound to magnetic beads according to the manufacturer's instructions. Culture of progenitor colonies was performed as previously described.<sup>22</sup> Individual colonies were lysed in RLT buffer (QIAGEN), and DNA was precipitated by the addition of isopropanol followed by centrifugation. All patients gave written informed consent for the research, which was approved by the Multi-Region Ethics Committee (Fulbourn) for the Causes of Clonal Haematological Disorders project, which permits the use of biologic samples and clinical data from patients with clonal hematologic disorders, and was carried out in accordance with the principles of the Declaration of Helsinki.

### Loss of heterozygosity and AML-associated genetic alterations

Genotyping of single nucleotide polymorphisms was performed by pyrosequencing (PV cohort) or direct sequencing (AML cohort). The *JAK2* mutation status of leukemic blasts was assessed by direct sequencing; samples in which the mutant allele was not detected were classified as *JAK2* wild-type, those with a 1:1 ratio of mutant to wild-type were classified as heterozygous, and those in which the wild-type allele was not detected or was less than 10% of the mutant allele were classified as homozygous. Mutations in *N/KRAS* (codons 12, 13, and 61), *CEBPA* (exon 1), *RUNX1* (all coding exons), *GATA2* (exon 4), *NPM* (exon 12), *WT1* (exons 7 and 9), *TP53* (exons 4-8), *CBL* (exons 8 and 9), and *TET2* (all coding exons) were assessed by direct sequencing. *FLT3*-ITD, *RUNX1-RUNX1T1*, and *CBFB-MYH11* alterations were assessed by polymerase chain reaction (PCR). Relative levels of *JAK2*, *EVII*, and *WT1* transcripts were measured in CD34<sup>+</sup> leukemic blasts, and nonmobilized peripheral blood CD34<sup>+</sup> cells from 4 healthy donors by SYBR green real-time PCR assays by using the  $\Delta\Delta C_t$  formula with *ABL1* as the housekeeping gene. Increased transcript levels were defined as greater than mean plus 2 standard deviations of the normal controls. *JAK2* copy number was assessed by real-time PCR, using a previously reported method<sup>23</sup> modified to include a second control region on chromosome 1p.

### Statistical analysis

Comparisons of diagnostic variables between patients were performed by using the *t* test for continuous variables and the Fisher exact test (for 2 × 2 tables).

## Results

### Association between *JAK2* mutation status of leukemic blasts and route of leukemic transformation

Sixteen patients with AML and evidence of a preceding *JAK2*-mutant MPN (15 *JAK2* V617F and 1 *JAK2* exon 12) were studied (Table 1). To ascertain the *JAK2* mutational status of each AML, it was important to acquire a sample of leukemic blasts free from contamination by the preceding MPN. Direct sequencing of leukemic blasts ( $\geq 90\%$  pure) showed 6 *JAK2* V617F-homozygous, 1 *JAK2* V617F-heterozygous, and 9 *JAK2* wild-type leukemias, the latter group included the single patient with a preceding *JAK2* exon 12 mutation (Table 1; supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Patients progressing to *JAK2*-mutant or wild-type leukemia did not differ with regard to sex ( $P = .6$ ), age at MPN diagnosis ( $P = .3$ ), prior use of cytoreductive therapy ( $P = .2$ ), or duration of the preceding MPN (*JAK2*-mutant AML,  $7 \pm 6$  years; *JAK2* wild-type AML,  $12 \pm 9$  years;  $P = .2$ ), although the relatively small sample size means that a genuine association may not have been detected. Patients with *JAK2* wild-type leukemia, however, were significantly older at time of progression to AML (*JAK2*-mutant AML,  $67 \pm 8$  years; *JAK2* wild-type AML,  $79 \pm 10$  years;  $P = .02$ ). These results contrast with a previous study wherein duration of the preceding MPN was shorter in those progressing to *JAK2* wild-type AML,<sup>10</sup> and await confirmation in a larger patient cohort.

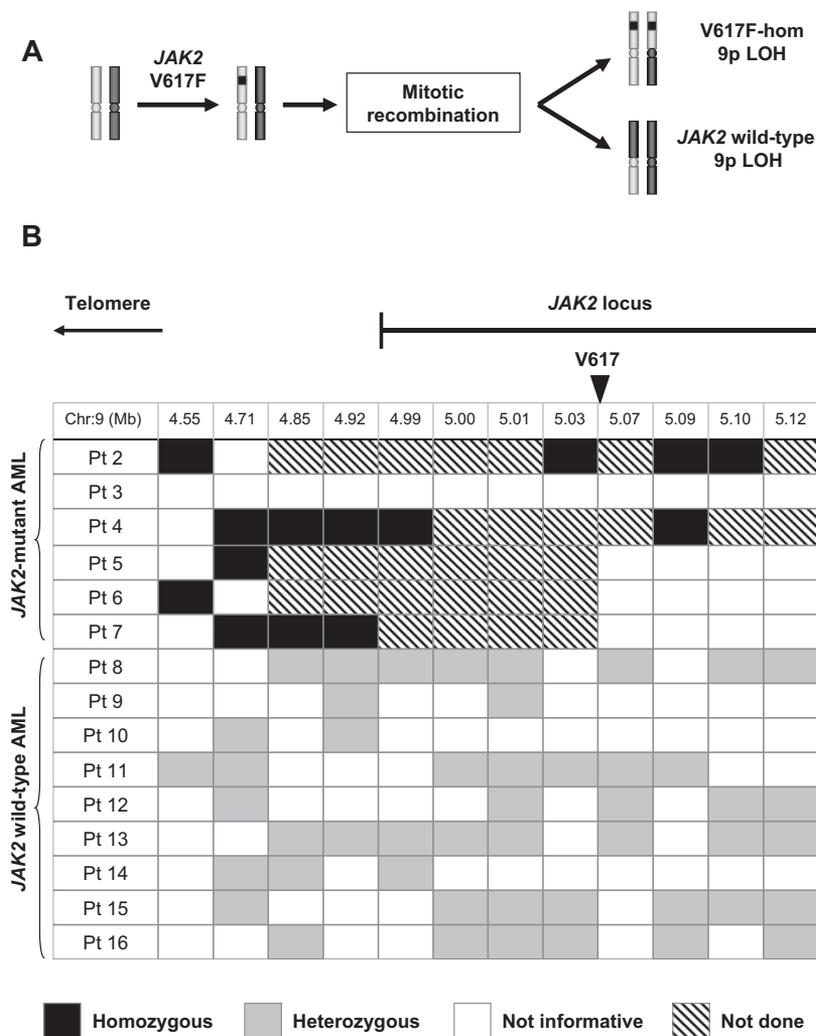
Of the 7 patients with *JAK2*-mutant AML, 6 had a well-characterized preceding MPN that fulfilled criteria for PMF<sup>20</sup> or myelofibrotic transformation of PV or ET.<sup>21</sup> The remaining patient with *JAK2*-mutant leukemia gave no history of overt prior blood disorder at the time of presentation with AML (patient 5, Table 1). However, the presence of splenomegaly palpable to the umbilicus together with a trephine biopsy showing clusters of hyperlobated megakaryocytes and dense reticulin fibrosis (supplemental Figure 2) indicated the presence of a preceding MPN, most likely PMF or myelofibrotic transformation of ET/PV. Therefore, all 7 transformations to *JAK2*-mutant AML arose either from myelofibrotic transformation of ET/PV or from PMF. In marked contrast, only 1 of 9 patients with *JAK2* wild-type AML had prior evidence of PMF or myelofibrotic transformation ( $P = .001$ ). In the remaining 8 cases leukemic transformation occurred directly from chronic-phase ET, PV, or RARS-T. In these 8 patients, blood counts 1 year before AML transformation all showed normal levels of hemoglobin ( $\geq 120$  g/L), white cells ( $4-9 \times 10^9/L$ ), and platelets ( $150-450 \times 10^9/L$ ) in the absence of constitutional symptoms or increasing splenomegaly. Taken together these data show an association between the *JAK2* status of leukemic blasts and the route by which leukemic transformation occurs: AML arising in patients with chronic-phase ET, PV, or RARS-T was generally *JAK2* wild-type, whereas *JAK2*-mutant leukemia was preceded by evidence of primary myelofibrosis or myelofibrotic transformation of ET/PV.

**Table 1. Features of 16 patients with progression to acute leukemia after a JAK2-mutant MPN**

Pt	Age at diagnosis, y/sex	MPN	Myeloproliferative neoplasm			JAK2 mutation			At transformation to AML			Altered gene expression
			Therapy	Disease duration, y	Sample from MPN phase	JAK2 mutation status	Cytogenetic analysis	Additional mutations				
1	63/M	PMF	HC, AN	2	BM at diagnosis	JAK2 V617F	Het	3 copies AML1*	NRAS G12S, RUNX1 D171N	—	—	
2	47/M	ET->MF	HC	16	Grans 1 y pre-AML	JAK2 V617F	Hom	inv(3), del(7)	NRAS G12D	↑ WT1, ↑ EVI1	↑ WT1, ↑ WT1	
3	47/F	PV->MF	HC, IFN	10	Grans 1 y pre-AML	JAK2 V617F	Hom	ND	RUNX1 N119K	↑ WT1	↑ WT1	
4	58/M	PMF	none	1	None	JAK2 V617F	Hom	ND	RUNX1 G138V	↑ WT1	↑ WT1	
5	70/M	Probable PMF	none	NA	NA	JAK2 V617F	Hom	add(8), del(7q)	RUNX1 D171N	↑ WT1	↑ WT1	
6	72/M	PMF	HC	8	None	JAK2 V617F	Hom	ND	—	↑ WT1, ↑ JAK2	↑ WT1, ↑ JAK2	
7	67/M	ET->MF	HC	6	Grans 1.5 y pre-AML	JAK2 V617F	Hom	der5, t(5;17)	TP53 R248Q	↑ WT1	↑ WT1	
8	43/M	PV	BU, P32, HC	26	None	JAK2 N542-E543del	Wild-type	del(20q)	TET2 D1242V, RUNX1 H215X	↑ WT1	↑ WT1	
9	79/M	PMF	HC	3	Mk 2 y pre-AML	JAK2 V617F	Wild-type	ND	CBL L380P	↑ WT1	↑ WT1	
10	75/F	PV	HC	12	None	JAK2 V617F	Wild-type	ND	FLT3-ITD, RUNX1 Q127X	NS	NS	
11	64/F	PV	HC	14	Blood 1 y pre-AML	JAK2 V617F	Wild-type	ND	TP53 S90X	NS	NS	
12	76/M	RARS-T	HC	8	None	JAK2 V617F	Wild-type	Complex†	TP53 V173M	↑ WT1	↑ WT1	
13	67/M	PV	BU, P32, HC	26	Blood 1 y pre-AML	JAK2 V617F	Wild-type	ND	TP53 C238F	↑ WT1	↑ WT1	
14	65/M	PV	HC	10	Blood 2 y pre-AML	JAK2 V617F	Wild-type	Complex‡	—	↑ WT1	↑ WT1	
15	58/F	PV	HC	3	Grans 2 y pre-AML	JAK2 V617F	Wild-type	add(9)	TET2 P1549X	—	—	
16	76/F	ET	HC, BU	10	Blood 0.5 y pre-AML	JAK2 V617F	Wild-type	ND	—	—	NS	

Pi indicates patient number; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; HC, hydroxycarbamide; AN, anagrelide; BM, bone marrow; het, heterozygous; AML, acute myeloid leukemia; ET, essential thrombocythemia; MF, myelofibrotic transformation; hom, homozygous; PV, polycythemia vera; IFN, interferon-α; ND, not done; NA, not applicable; Grans, granulocytes; BU, megakaryocytes; NS, no sample available; and RARS-T, refractory anemia with ringed sideroblasts and thrombocytosis.

\*Detected by fluorescence in situ hybridization; G-banding failed.  
 †43;XY,del(5)(q1q3),-7,-12,-16 [5],43,idelm,del(6)(q1q2) [3],46,XY [2].  
 ‡45-46,XY,del(5)(q1?),add(16)(q2?),-17,-17,-22,-22,-2-3 mar [10].



**Figure 1. Reversion to wild-type is not a common mechanism of progression from a *JAK2*-mutant myeloproliferative neoplasm to a *JAK2* wild-type leukemia.** (A) Mitotic recombination in a *JAK2* V617F-heterozygous cell gives rise to both V617F-homozygous and *JAK2* wild-type daughter cells, both of which harbor LOH for chromosome 9p. (B) Summary of SNP analysis in patients evolving to acute leukemia after a *JAK2*-mutant MPN, showing SNP locations relative to the *JAK2* locus and genotyping results, whereas all informative *JAK2* V617F-homozygous leukemias showed LOH close to *JAK2*, this was not observed in any of 9 *JAK2* wild-type leukemias. Patient 1 is not included in this analysis because leukemic blasts were heterozygous for the *JAK2* V617F mutation. SNP indicates single nucleotide polymorphism; hom, homozygous; and LOH, loss of heterozygosity.

### Progression to *JAK2* wild-type AML does not reflect mitotic recombination, gene conversion, or deletion of the mutant allele

It is unclear why patients with a *JAK2*-mutant MPN may develop a *JAK2* wild-type AML. A proportion of patients with MPN harbor a clone in which the mutant *JAK2* allele is duplicated and the wild-type allele is lost.<sup>1,2</sup> Such clones result from mitotic recombination in a V617F-heterozygous cell, which would produce V617F-homozygous and *JAK2* wild-type daughter cells (Figure 1A). Clonal expansion of the *JAK2* wild-type daughter cell could potentially give rise to a subsequent *JAK2* wild-type leukemia.

To search for evidence that mitotically recombined *JAK2* wild-type clones may persist in vivo, 1338 individual erythroid colonies from 10 patients with chronic-phase PV were genotyped for both the *JAK2* V617F mutation and single nucleotide polymorphisms (SNPs) within or telomeric to the *JAK2* locus. All 76 V617F-homozygous colonies showed loss of heterozygosity (LOH) consistent with prior mitotic recombination. LOH was not identified, however, in any of 1195 *JAK2*-wild type colonies (Table 2), indicating that the mitotically recombined *JAK2* wild-type daughter clone does not commonly expand to detectable levels in vivo.

The preceding data do not exclude the persistence of a very low level of recombined *JAK2* wild-type cells or the presence of such clones in a minority of patients, either of which could still explain the phenomenon of *JAK2* wild-type AML. To address this possibil-

ity, purified leukemic blasts from patients with AML transformation were investigated for LOH with the use of multiple SNPs within or adjacent to the *JAK2* locus. All 5 informative *JAK2* V617F-homozygous leukemias showed LOH telomeric to the *JAK2* locus, consistent with prior mitotic recombination affecting the *JAK2* locus and extending to the telomere, as previously reported.<sup>1,2</sup> However, no evidence for LOH was found in any of the 9 *JAK2* wild-type leukemias. All *JAK2* wild-type leukemias were heterozygous for at least one SNP telomeric to *JAK2*, 8 of 9 were heterozygous within the *JAK2* locus for SNPs telomeric to V617, and 6 of 9 were heterozygous within the *JAK2* locus for SNPs centromeric to V617 (Figure 1B; supplemental Figure 1).

Taken together, these data indicate that in PV the *JAK2* wild-type daughter cell resulting from mitotic recombination does not commonly expand to detectable levels in vivo. Moreover, after AML transformation, *JAK2* wild-type leukemic blasts do not reflect loss of the *JAK2* mutation by mitotic recombination, gene conversion, or gene deletion.

### Genetic lesions associated with progression to AML include alterations of *RUNX1*, *TP53*, *NRAS*, *EVI1*, and *WT1*

Progression from MPN to AML is probably associated with the acquisition of further genetic events. To ascertain whether differences exist between *JAK2*-mutant and *JAK2* wild-type leukemia in

**Table 2. Genotyping of individual erythroid colonies from patients with polycythemia vera for 9p loss of heterozygosity**

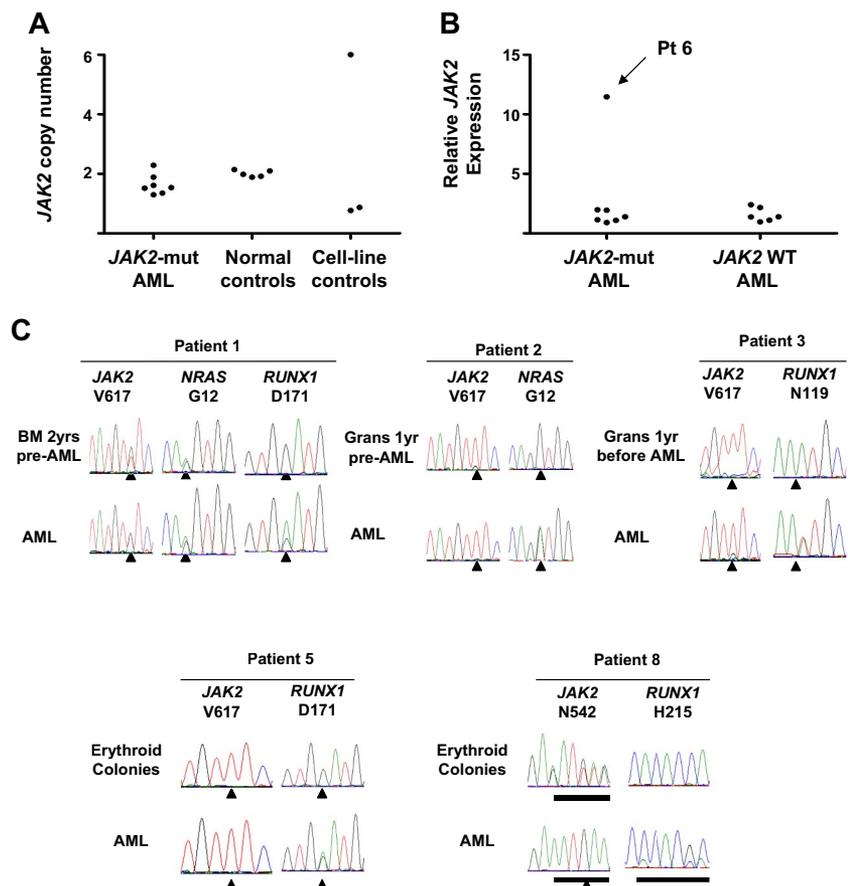
Patient	9p SNP	<i>JAK2</i> wild-type		<i>JAK2</i> V617F-het		<i>JAK2</i> V617F-hom	
		9p het	9p LOH	9p het	9p LOH	9p het	9p LOH
a	rs465514	160	0	0	0	0	5
b	rs428111	154	0	19	0	0	5
c	rs428111	56	0	2	0	0	2
d	rs428111	78	0	9	0	0	6
e	rs2230724	64	0	3	0	0	5
f	rs428111	58	0	3	0	0	1
g	rs428111	64	0	5	0	0	1
h	rs7847141	119	0	15	0	0	1
i	rs428111	334	0	2	0	0	49
j	rs428111	108	0	9	0	0	1
Total		1195	0	67	0	0	76

Patients a-j represent a distinct cohort from those transforming to AML (patients 1-16 in Tables 1 and 3). Individual erythroid colonies were genotyped by pyrosequencing for both the *JAK2* V617F mutation and an informative SNP on 9p within or telomeric to *JAK2*. Data are the number of colonies of each individual genotype. All *JAK2* wild-type colonies were heterozygous for a 9p SNP.

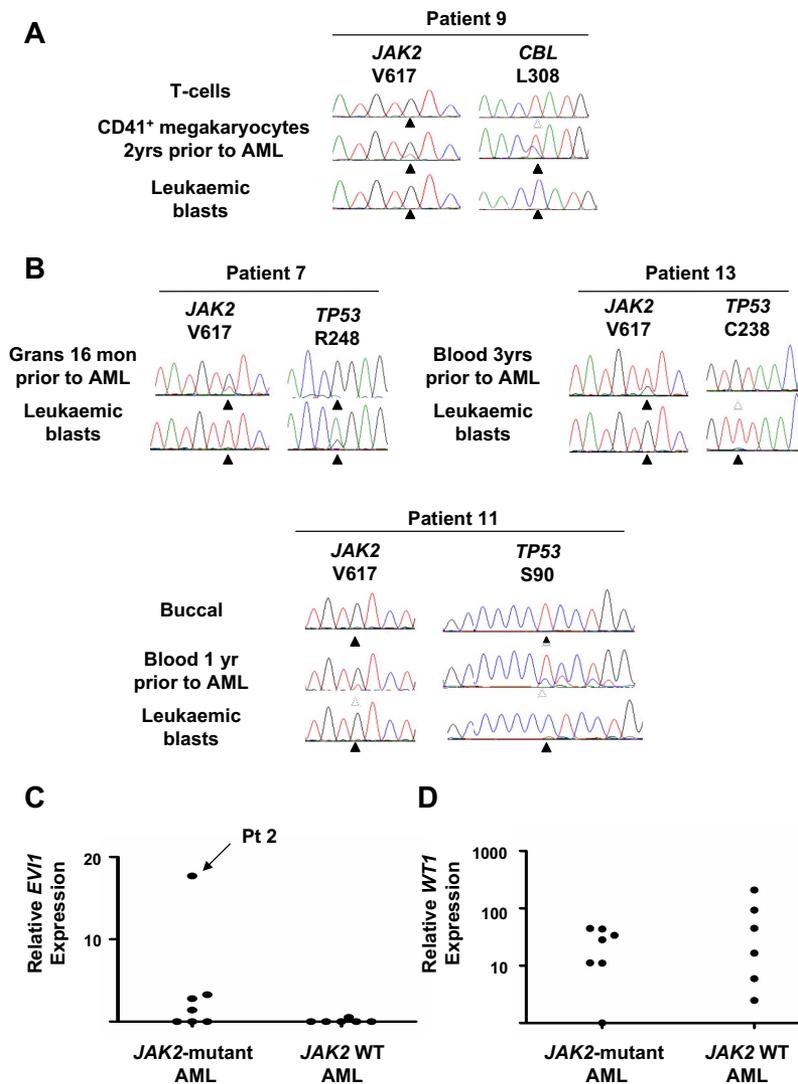
SNP indicates single nucleotide polymorphism; het-, heterozygous; hom-; homozygous; and LOH, loss of heterozygosity.

the pattern of genetic lesions, patients were screened for aberrations known to be involved in AML pathogenesis. Because *BCR-ABL1* copy number and transcript levels may increase in the blastic phase of chronic myeloid leukemia,<sup>24</sup> *JAK2* copy number and transcript levels were assessed. No increase in *JAK2* copy number was observed in any of 7 *JAK2*-mutant leukemias (Figure 2A). RNA from CD34<sup>+</sup> leukemic blasts was available from all 7 *JAK2*-mutant and from 6 of 9 *JAK2* wild-type leukemias. Analysis showed a 12-fold increase in *JAK2* transcripts in one *JAK2* V617F-homozygous leukemia relative to normal CD34<sup>+</sup> cells (patient 6, Figure 2B) despite a normal *JAK2* copy number (Figure 2A), suggesting that elevated *JAK2* expression may play a role in disease progression in a proportion of patients.

Leukemic blasts were assessed for alterations in known leukemia-associated genes (*N/KRAS*, *CEBPA*, *RUNX1*, *GATA2*, *NPM*, *WT1*, *FLT3*, *CBL*, *EVII*, and *TP53*). Two of 7 *JAK2*-mutant leukemias harbored oncogenic *RAS* mutations, and in one case the mutation was present 2 years before transformation (patients 1 and 2, Table 1; Figure 2C). Mutations in *CBL* or *FLT3* were identified in single patients with *JAK2* wild-type leukemia (patients 9 and 10, respectively, Table 1) and the *CBL* mutation was also detected in megakaryocytes 2 years before AML transformation (Figure 3A). Mutations in *RUNX1* were identified in 2 of 9 *JAK2* wild-type and 4 of 7 *JAK2*-mutant leukemias (Table 1). In all cases *RUNX1* mutations were acquired and the wild-type allele was retained. In 4 patients in whom material representative of the preceding MPN



**Figure 2. Genetic events associated with progression to acute myeloid leukemia after a *JAK2*-mutant MPN.** (A) Assessment of *JAK2* copy number in 7 *JAK2*-mutant leukemias with the use of a real-time PCR assay; samples from healthy persons and cell lines known to harbor a deletion of 9p (MDA-MB-361 and NB16) or multiple copies of *JAK2* (HEL) are shown as controls. (B) Expression of total *JAK2* measured by real-time PCR normalized to normal CD34<sup>+</sup> cells showing a 12-fold increase in relative *JAK2* expression in patient 6. (C) Mutation genotyping from 5 patients progressing to acute leukemia showing that, whereas *RUNX1* mutations were acquired at the time of progression to AML, *NRAS* mutations may be associated with the preceding MPN. Mut indicates mutant; WT, wild-type; grans, purified granulocytes; and BM, bone marrow.



**Figure 3. *CBL* and *TP53* mutations and *EVI1* and *WT1* expression in patients progressing to acute leukemia.** (A) *CBL* mutation in patient 9 showing detection of the mutation in CD41<sup>+</sup> megakaryocytes 2 years before AML transformation and loss of the wild-type *CBL* allele in leukemic blasts. (B) *TP53* mutations in 3 patients progressing to acute leukemia showing the presence of the mutation 1 year before transformation in patient 11. (C) *EVI1* expression measured by real-time PCR normalized to normal CD34<sup>+</sup> cells showing a 17-fold increase in relative *EVI1* expression in patient 2. (D) *WT1* expression measured by real-time PCR normalized to normal CD34<sup>+</sup> cells showing increased transcript levels (>2 standard deviations above the mean of the normal controls) in 6 of 7 *JAK2*-mutant and 5 of 6 *JAK2* wild-type leukemias. Grans indicates purified granulocytes; and mon, months.

was available (patients 1, 3, 5, and 8), *RUNX1* mutations were undetectable in either bone marrow, peripheral blood granulocytes, or erythroid colonies (Figure 2C). In patient 5, leukemic blasts harbored mutations in both *JAK2* and *RUNX1*, but erythroid colonies were all *RUNX1* wild-type, with or without a *JAK2* mutation (*JAK2* V617F-homozygous, n = 43; *JAK2* wild-type, n = 3; Figure 2C), providing further evidence for an established *JAK2* V617F-positive clone in this patient before leukemic transformation.

Mutations leading to alterations in the DNA binding domain of p53 were detected in 1 of 7 *JAK2*-mutant and 3 of 9 *JAK2*-wild-type leukemias (Table 1). In all cases the mutations were acquired and showed complete or partial loss of the wild-type allele in leukemic blasts (Figure 3B). In 2 of these patients, *TP53* mutations were undetectable in the preceding MPN (patients 7 and 13), although in a further patient (patient 11) a *TP53* mutation could be detected in peripheral blood obtained 1 year before AML transformation (Figure 3B), at which time the patient was well and the full blood count was normal.

A single *JAK2*-mutant leukemia showed a 17-fold increase in *EVI1* transcript levels, consistent with an inversion of chromosome 3 detected at leukemic transformation (patient 2, Table 1), with the remaining AML samples showing either no expression of *EVI1* or expression comparable with normal CD34<sup>+</sup> cells (Figure 3C).

Increased *WT1* transcript levels were present in most cases, with only 1 *JAK2*-mutant and 1 *JAK2* wild-type leukemia showing transcript levels comparable with normal CD34<sup>+</sup> cells (Figure 3D).

In summary, some leukemia-associated mutations were found only at the time of AML transformation (eg, *RUNX1*) and were not detected in the preceding MPN, even when this consisted of PMF or myelofibrotic transformation of ET/PV. By contrast other mutations were detectable before leukemia transformation (eg, *TP53* and *CBL*). No consistent differences were observed in the pattern of genetic alterations present in leukemias positive or negative for *JAK2* mutations.

#### Clonal analysis of additional genetic lesions: *JAK2* wild-type AML may harbor *TET2* mutations that do not precede acquisition of a *JAK2* mutation

Clonal analysis was used to explore the phylogenetic relationship between *JAK2* wild-type leukemia and a preceding *JAK2*-mutant MPN. Individual hematopoietic colonies from 4 patients with *JAK2* wild-type AML were studied with the use of analysis of X-chromosomal inactivation patterns or the detection of additional genetic alterations (Table 3).

Samples of RNA from both the *JAK2*-mutant MPN and *JAK2* wild-type AML were available from a single female patient (patient

**Table 3. Clonal analysis of individual erythroid colonies and purified leukemic blasts from 4 patients progressing to *JAK2* wild-type leukemia**

Patient	Additional mutation	Erythroid colonies		<i>JAK2</i> wild-type leukemic blasts
		<i>JAK2</i> mutant	<i>JAK2</i> wild-type	
8	del(20q)	+	-	-
8	<i>TET2</i>	-	+	+
8	<i>RUNX1</i>	-	-	+
9	<i>CBL</i>	-	-	+
12	<i>TP53</i>	-	-	+
15	add(9)	+	-	-
15	<i>TET2</i>	-	-	+

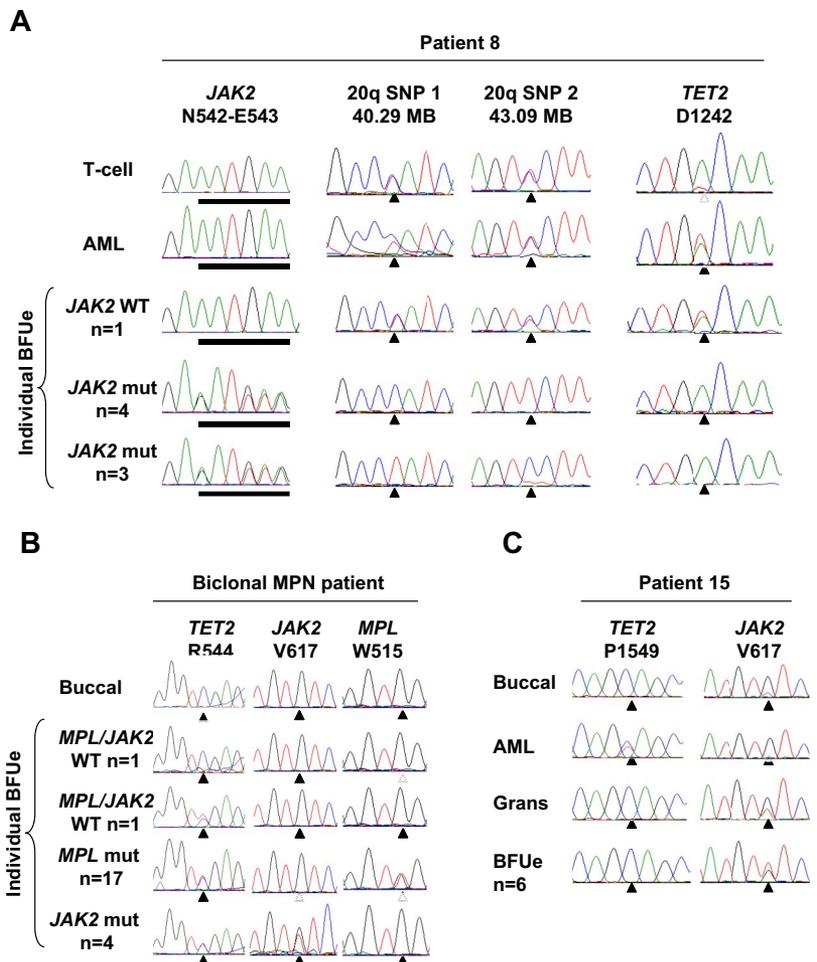
+ Indicates present; and -, absent.

15, Table 1). In this patient, the same X-chromosome inactivation pattern was observed in *JAK2* V617F-positive erythroid colonies, peripheral blood granulocytes obtained 2 years before transformation, and *JAK2* wild-type leukemic blasts (supplemental Figure 3A), a result that does not discriminate between a shared versus independent clonal origin for *JAK2*-mutant MPN and *JAK2* wild-type AML.

Individual hematopoietic colonies were available from 2 patients in whom cytogenetic abnormalities were detected at transformation to *JAK2* wild-type AML (patients 8 and 15, Tables 1 and 3). In patient 15, trisomy 9 was present in 9 of 10 metaphases at AML transformation and was also detected in *JAK2*-mutant erythroid colonies (as previously described in Beer et al<sup>19</sup>) but was absent from *JAK2* wild-type leukemic blasts as shown by a 1:1 allelic ratio

of multiple informative SNPs within the *JAK2* locus (supplemental Figure 1; data not shown). In patient 8 a deletion of chromosome 20q was present in 9 of 14 metaphases at progression to AML and was also present in *JAK2*-mutant erythroid colonies as shown by LOH studies but was absent from *JAK2* wild-type leukemic blasts (Table 3). Consistent with a recent report,<sup>17</sup> different *JAK2*-mutant erythroid colonies harbored loss of alternative 20q alleles (Figure 4A), indicating the occurrence of at least 2 independent 20q deletions in this patient. In these 2 patients, therefore, cytogenetic aberrations detected at AML transformation reflected cells derived from the preceding *JAK2*-mutant MPN rather than from the *JAK2* wild-type leukemia. Hematopoietic colonies were also available from 2 patients with mutations in *CBL* or *TP53* (patients 9 and 12, Tables 1 and 3). In these patients, *CBL* or *TP53* mutations were present in *JAK2* wild-type leukemic blasts but were absent from *JAK2*-mutant erythroid colonies (Table 3). In summary, in these 4 patients the detection of additional genetic events did not indicate the existence of a shared clone of origin for *JAK2*-mutant MPN and *JAK2* wild-type AML.

Mutations in *TET2* have recently been described in patients with a chronic-phase *JAK2*-mutant MPN and may precede acquisition of the *JAK2* mutation.<sup>18,25</sup> Sequencing of the *TET2* coding region identified an acquired mutation (*TET2* R544X) in 1 of 3 patients with a biclonal chronic-phase MPN. Analysis of individual erythroid colonies from this patient identified the *TET2* mutation in *JAK2*-mutant, *MPL*-mutant, and *JAK2/MPL* wild-type colonies (Figure 4B), showing that the *JAK2*-mutant and *MPL*-mutant clones had evolved as subclones from a shared *TET2*-mutant



**Figure 4. Clonal relationship of *JAK2* wild-type leukemia to *JAK2*-mutant MPN with the use of 20q deletion or mutation in *TET2* as a clonal marker. (A) Analysis of patient 8 showing a deletion of alternative 20q alleles in the MPN clone but no 20q deletion in the AML clone and a mutation of *TET2* in leukemic blasts and a *JAK2* wild-type erythroid colony but not in *JAK2*-mutant erythroid colonies. (B) Analysis of individual erythroid colonies from a patient harboring *JAK2* V617F and *MPL* W515L mutations, showing that mutations in *JAK2* and *MPL* are present in separate clones, with both clones have arisen from a shared *TET2*-mutant founder clone. (C) Analysis of patient 15 showing a mutation of *TET2* in the *JAK2* wild-type leukemia but not the *JAK2*-mutant MPN.**

proliferation. These results show that a *TET2*-mutant founder clone may give rise to daughter clones bearing different additional genetic alterations.

It is possible, therefore, that a *TET2*-mutant clone could give rise to both *JAK2*-mutant MPN and *JAK2* wild-type AML within the same patient. Within the AML cohort, 2 patients with *JAK2* wild-type leukemia harbored acquired mutations in *TET2* (patients 8 and 15, Table 1). To investigate the possibility that a *JAK2*-mutant MPN and *JAK2* wild-type AML may also arise from a shared *TET2*-mutant founder clone, individual erythroid colonies from both patients were genotyped for the *TET2* mutation. In patient 8, a *TET2* mutation was present in leukemic blasts and a *JAK2* wild-type erythroid colony but was absent from all 7 *JAK2*-mutant erythroid colonies (Figure 4A; Table 3). Similarly in patient 15, a *TET2* mutation was present in leukemic blasts but was not detected in granulocytes obtained 2 years before transformation and was absent from all 6 *JAK2*-mutant erythroid colonies (Figure 4C; Table 3). Thus, in both patients *TET2* mutations were present only in the *JAK2* wild-type leukemia and not in the preceding *JAK2*-mutant MPN. Of note, the presence of a *TET2*-mutant erythroid colony in a patient with *TET2*-mutant AML (patient 8) implies that the *TET2* mutation was acquired early in disease evolution, before the acquisition of a fully transformed AML phenotype. We considered the possibility that the *TET2* and *JAK2* mutations were acquired within the same clone, with mitotic recombination or deletion of *TET2* leading to loss of the mutant *TET2* allele within the *JAK2*-mutant clone. This possibility was excluded, however, by the detection of a heterozygous SNP within the *TET2* locus in *JAK2*-mutant erythroid colonies and *JAK2* wild-type leukemic blasts from both patients (supplemental Figure 3B). Our data therefore indicate that the *TET2* mutation did not precede acquisition of the *JAK2* mutation in these 2 patients and show that mutations in *TET2* and *JAK2* may be present in separate clonal expansions.

## Discussion

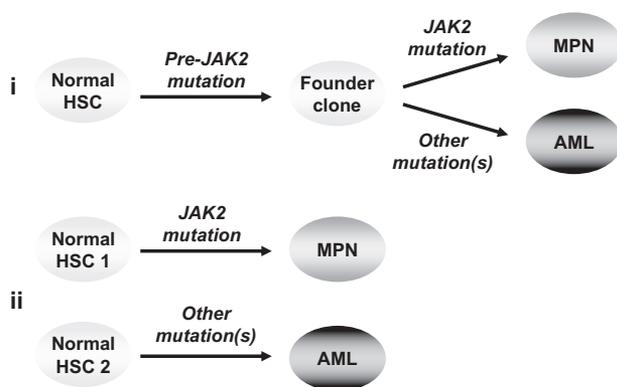
This study used analysis of purified leukemic blasts from 16 patients to address several issues relating to AML transformation after a *JAK2*-mutant MPN, including the relationship of MPN phenotype to leukemia *JAK2* status, the nature of the genetic lesions acquired with progressive disease, and the clonal relationship of *JAK2*-mutant MPN to *JAK2* wild-type AML. Analysis of a pure population of leukemic blasts is critical to establish whether the *JAK2* mutation or additional genetic lesions are present within the leukemic clone or in cells representing the preceding MPN. The culture of erythroid colonies from selected patients provided valuable clonal material representing the *JAK2*-mutant MPN, thus permitting the precise tracking of mutations and cytogenetic aberrations through the MPN and AML phases of disease. The importance of obtaining purified leukemic blasts and clonal MPN-phase material is highlighted by the demonstration in 2 patients that cytogenetic abnormalities found in most cells at AML transformation indicated persistence of the *JAK2*-mutant MPN and were absent from *JAK2* wild-type leukemic blasts.

In this study, a striking correlation was observed between the phenotype of the preceding MPN and the *JAK2* status of leukemic blasts. In contrast to *JAK2* wild-type AML, evolution to *JAK2*-mutant leukemia was invariably preceded by myelofibrotic transformation of ET/PV or PMF. It has been suggested that myelofibrotic transformation of ET/PV and at least some cases of PMF may both

represent manifestations of accelerated phase disease.<sup>1</sup> In support of this notion, PMF and post-ET/PV myelofibrosis are phenotypically indistinguishable,<sup>26,27</sup> and PMF is associated with features of advanced disease, including a high prevalence of genomic and epigenetic alterations,<sup>28-30</sup> progressive stem cell dysfunction,<sup>31,32</sup> and shortened overall survival.<sup>5</sup> Our results therefore imply that evolution to *JAK2*-mutant AML is preceded by the acquisition of genetic changes that give rise to the phenotype of accelerated-phase disease, manifested clinically as PMF or myelofibrotic transformation of ET/PV. Mutant *JAK2* may well be responsible for this accumulation of DNA damage. Bcl-X<sub>L</sub> deamidation in response to DNA damage is inhibited in *JAK2* V617F-positive primary cells, thus providing a mechanism for the aberrant survival of DNA-damaged cells.<sup>7</sup> Moreover, expression of *JAK2* V617F in cell lines and human progenitors is associated with both increased DNA damage and aberrant DNA repair.<sup>6</sup>

In contrast to *JAK2*-mutant AML, *JAK2* wild-type leukemia usually arose in patients with chronic-phase ET or PV. Although not statistically significant, it is noteworthy that all 3 patients with a history of prior genotoxic therapy (busulphan or P32) progressed to *JAK2* wild-type AML (Table 1). It is possible that leukemic transformation in such cases represents therapy-associated damage to a normal stem cell. In the remaining 6 *JAK2* wild-type AMLs, the patients had received hydroxycarbamide alone. Concerns have been raised over a possible increased risk of AML in patients treated with hydroxycarbamide, and an association between hydroxycarbamide therapy and alteration of *TP53* has been suggested.<sup>33,34</sup> Deletion or mutation of *TP53* was seen in the current study in 4 patients treated with single-agent hydroxycarbamide, but both these data and previous reports lack a hydroxycarbamide-free control group. It is clear that leukemic transformation does occur in the absence of prior therapy,<sup>4,10</sup> and an increased risk with hydroxycarbamide has been difficult to establish, with clinical studies giving conflicting results.<sup>4,34-37</sup> Follow-up of patients with sickle cell suggests that hydroxycarbamide is not leukemogenic to normal stem cells,<sup>38</sup> although these data do not exclude a mutagenic effect on clonal hematopoiesis (eg, a pre-*JAK2* clone). We have previously shown that patients with *JAK2* V617F-positive ET are more sensitive to the effects of hydroxycarbamide than are patients without the mutation,<sup>39</sup> raising the possibility that hydroxycarbamide might preferentially suppress the *JAK2*-mutant clone and favor expansion of coexisting *JAK2* wild-type clones with subsequent leukemic transformation of the latter. However, at present, the data implicating hydroxycarbamide in progression to AML remain inconclusive.

To gain insight into the mechanisms underlying the development of *JAK2* wild-type AML, we studied its clonal relationship to the preceding *JAK2*-mutant MPN. In all 9 cases examined we were able to exclude the possibility that *JAK2* wild-type AML arose from the *JAK2* wild-type progeny of mitotic recombination affecting a *JAK2* V617F-heterozygous cell (Figure 1A), with analysis of multiple markers within the *JAK2* gene also excluding intrachromosomal deletion or gene conversion. These data confirm and considerably extend 3 previously reported cases, in which reversion to wild-type by mitotic recombination was excluded by analysis of a limited number of markers.<sup>9,10</sup> Moreover in this study, the persistence of mitotically recombined *JAK2* wild-type clones was not detected by analysis of more than 1300 individual erythroid colonies from 10 patients with chronic-phase PV. Taken together, these data indicate that loss of the mutant *JAK2* allele by mitotic recombination is unlikely to be a significant mechanism in the genesis of *JAK2* wild-type AML.



**Figure 5. Models to explain progression from a *JAK2*-mutant MPN to a *JAK2* wild-type leukemia.** In model 1 (i) the 2 phases of disease are phylogenetically related, having arisen from a shared (pre-*JAK2*) founder clone, whereas in model 2 (ii) the 2 phases of disease are clonally unrelated, reflecting transformation of independent stem cells. HSC indicates hematopoietic stem cell.

Two models remain to explain the clonal relationship of *JAK2* wild-type AML to its preceding *JAK2*-mutant MPN: (1) the 2 phases of disease are phylogenetically related, having arisen from a shared (pre-*JAK2*) founder clone, or (2) the MPN and AML are clonally unrelated and reflect transformation of independent stem cells (Figure 5). In the present study, oligoclonal disease was shown in 4 patients transforming to *JAK2* wild-type AML, with mutations in *TP53*, *CBL*, or *TET2* present in a clone distinct from that carrying the *JAK2* mutation; however, the phylogenetic relationship of the 2 clones remains unclear. Of note, in 2 of these cases progression to *TET2*-mutant AML did not reflect transformation of a pre-*JAK2* clone carrying the *TET2* mutation. It remains theoretically possible, however, that in these 2 patients the *JAK2*-mutant MPN and *TET2*-mutant AML represent the phylogenetically related progeny of a shared pre-*TET2* clone.

Recent reports have uncovered an increasing degree of clonal heterogeneity in patients with MPN. Mutations in *TET2* may precede acquisition of a *JAK2* mutation,<sup>18</sup> and data presented herein show that genetically dissimilar clones, bearing mutations in *JAK2* or *MPL*, represent the phylogenetically related progeny of a shared *TET2*-mutant founder clone in a patient with chronic-phase ET. By contrast, we have previously shown that biclonal disease may represent transformation of distinct, unrelated stem cells.<sup>19</sup> Further evidence of oligoclonal disease in patients in chronic phase includes deletion of alternative chromosome 20q alleles<sup>40</sup> (and present study), the acquisition of multiple *JAK2* V617F mutations in individual patients,<sup>41</sup> and the detection in patients with CML treated with imatinib of cytogenetically abnormal *BCR-ABL1*-negative clones.<sup>42</sup> Clonal heterogeneity has also been detected in epithelial malignancy, with molecular analysis of multifocal tumors showing that clones with diverse patterns of somatic mutation represent either the phylogenetically related progeny of a shared ancestral clone or transformation of unrelated progenitor cells.<sup>43-45</sup>

Thus, oligoclonal disease is present in a proportion of patients with myeloid or epithelial malignancy, composed of either phylogenetically related or unrelated clones (akin to models 1 and 2 in Figure 5). Given these observations, it seems plausible that each model may be operating in a different subset of patients with *JAK2* wild-type AML.

The presence of clonal heterogeneity in both myeloid and epithelial neoplasia implies that early tumorigenesis is characterized by the proliferation of competing clones harboring diverse patterns of mutation, with clonal dominance arising through a Darwinian process of biologic selection. The preleukemic MPNs offer an experimentally tractable system for the study of early-stage malignancy through the precise tracking of mutations within different clonal expansions. The identification of novel mutations has facilitated the detection of an increasing degree of clonal complexity in patients with MPN. Given that only a limited number of MPN-associated mutations have so far been identified, it is probable that phenomena such as biclonal disease and pre-*JAK2* clonal expansions are more prevalent than is currently appreciated.

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## Authorship

Contribution: P.A.B. designed and performed experiments, analyzed data, and co-wrote the manuscript; F.D., J.-P.L.C., and W.V. performed and analyzed sequencing of *TET2*; M.A.D. and E.C. performed colony assays; D.B., R.K., M.F.M., C.N.H., and A.M.V. provided patient samples and clinical information; and A.R.G. directed the research and co-wrote the manuscript. All authors had the opportunity to review the manuscript.

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## Two routes to leukemic transformation after a *JAK2* mutation–positive myeloproliferative neoplasm

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