Leukemic blasts in transformed JAK2-V617F positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation

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Running title: JAK2 in secondary AML
Abstract

To study the role of the JAK2-V617F mutation in leukemic transformation, we examined 27 patients with myeloproliferative disorders (MPD) who transformed to acute myeloid leukemia (AML). At MPD diagnosis, JAK2-V617F was detectable in 17/27 patients. Surprisingly, only 5/17 patients developed JAK2-V617F positive AML, whereas 9/17 patients transformed to JAK2-V617F negative AML. Microsatellite analysis in a female patient showed that mitotic recombination was not responsible for the transition from JAK2-V617F positive MPD to JAK2-V617F negative AML, and clonality determined by the MPP1-polymorphism demonstrated that the granulocytes and leukemic blasts inactivated the same parental X-chromosome. In a second patient positive for JAK2-V617F at transformation, but with JAK2-V617F negative leukemic blasts, we found del(11q) in all cells examined, suggesting a common clonal origin of MPD and AML. We conclude that JAK2-V617F positive MPD frequently yields JAK2-V617F negative AML and transformation of a common JAK2-V617F negative ancestor represents a possible mechanism.

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Introduction

Myeloproliferative disorders (MPD) are a heterogeneous group of diseases characterized by increased hematopoiesis leading to elevated numbers of non-lymphoid cells and/or platelets in the peripheral blood. An acquired somatic mutation in the JAK2 gene resulting in a valine to phenylalanine substitution at position 617 (JAK2-V617F) is frequently found in patients with MPDs.\textsuperscript{1-4} Using a sensitive allele-specific PCR assay, the JAK2-V617F mutation is detectable in >90% of patients with polycythemia vera (PV), >50% of essential thrombocytemia (ET) and >50% of idiopathic myelofibrosis (IMF).\textsuperscript{2,5,6} Transformation to AML is a known complication of MPDs.\textsuperscript{7-9} The frequency of JAK2-V617F in patients with de novo AML was around 1%.\textsuperscript{10-15} In contrast, the JAK2-V617F mutation in AML secondary to MPD was in the range of 50%.\textsuperscript{12,14,16} The question of whether the presence of the JAK2-V617F mutation favors leukemic transformation is currently unanswered. Due to the small number of patients with secondary AML, prospective studies are difficult to perform and the data is at present inconclusive.\textsuperscript{17} Here we compared the JAK2-V617F status before and at leukemic transformation in 27 MPD patients and performed molecular studies on purified cell populations.
Patients, Materials and Methods

Patients and samples
We performed a retrospective study on 27 patients with AML secondary to MPD (8 PV, 12 ET, 7 IMF) from whom samples at MPD and AML diagnosis were available. Informed consent from all patients alive was obtained according to the Declaration of Helsinki. The study was conducted according to the guidelines of the local ethics committees at centers in France (Nantes, Dijon, Bordeaux) and Switzerland (Basel). The diagnosis of MPD was made according to the criteria of the World Health Organization (patients from Basel) or of the Polycythemia Vera Study Group (Nantes, Dijon, Bordeaux).  

Cell separations, RNA and DNA isolation
Bone marrow or peripheral blood smears were scraped and DNA was isolated using the QIAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Leukemic blastss were isolated by fluorescence activated cell sorting (FACS) using side scatter and anti-CD34 antibodies or by laser capture microdissection of bone marrow films stained with May-Grünwald/Giemsa. Cells of the granulocytic lineage and T cells were sorted by FACS using anti-CD15 and anti-CD3 antibodies, respectively. Purity was > 95%. Preparations of DNA and RNA and cDNA syntheses were performed as described.  

Molecular analyses
The methods for detecting loss of heterozygosity on chromosome 9p (9pLOH) and for determining the copy number of chromosome 9p were described earlier. A quantitative PCR assay for JAK2-V617F was performed with two different methods, and analysis of clonality by X-chromosome inactivation in female patients heterozygous for a single nucleotide polymorphism in the MPP1 gene was assessed as described.

Statistical analysis
To compare continuous variables among the groups we used the Mann-Whitney U-test.
Results and Discussion

We determined the JAK2-V617F mutation status by allele-specific PCR (AS-PCR) in 27 MPD patients (8 PV, 12 ET, and 7 IMF) from whom we obtained paired DNA samples at MPD diagnosis and leukemic transformation. At MPD diagnosis, the JAK2-V617F mutation was present in 17 of 27 patients (63%) (Table 1). Surprisingly, in 7 of these 17 patients the JAK2 mutation was undetectable in unfractionated peripheral blood or bone marrow samples at transformation to AML. Our AS-PCR assays have a detection limit of <1%T.6,22 In two additional patients we found that leukemic blast cells purified by FACS (patient DI-35) or laser capture microdissection (patient NA-48) were negative for JAK2-V617F, although the JAK2 mutation was detectable in unfractionated AML samples (Table 1). Thus, 9/17 patients (53%) with JAK2-V617F positive MPD displayed JAK2-V617F negative leukemic blasts at diagnosis of AML. Purified granulocytes or CD15-positive myeloid cells obtained in 6 of these 9 patients were also negative for the JAK2 mutation, suggesting that the AML clone exerted a suppressive effect on the JAK2-V617F positive MPD clone (Table 1). In 5 patients (29%) we found JAK2-V617F both at MPD diagnosis and in leukemic blasts at transformation. In these 5 patients the allelic ratio of JAK2-V617F in blasts was greater than 50%T suggesting that at least a proportion of blasts were homozygous for JAK2-V617F. In the remaining 3/17 patients (18%) with JAK2-V617F positive MPD, the mutational status of the AML blasts could not be determined with certainty, due to lack of purified cells.

Patients positive for JAK2-V617F at MPD diagnosis who transformed to JAK2-V617F negative AML had a markedly shorter interval between diagnosis of MPD and leukemic transformation than those who transformed to JAK2-V617F positive AML (3±2 versus 10±7 years, n=14, p=0.013) (see supplemental Table S1). Information on treatment during the MPD phase was available in 25/27 patients (93%). Two patients transformed to AML without cytoreductive treatment, whereas the remaining patients received hydroxyurea (15), pipobroman (6) or other cytoreductive drugs (Table 1).
To address the question whether the MPD and AML clones in these patients arose de novo from different progenitor or stem cells, or represent subclones derived from a common ancestor, we performed molecular analyses in two cases. In one female patient with PV (NA-02), we compared clonality by 9pLOH and X-chromosommal inactivation pattern (XCIP) in purified cell populations at both stages of the disease (Figure 1). At diagnosis of MPD, this patient had 94%T and displayed 9pLOH (Figure 1A). Deletion of chromosome 9p was excluded by determining the copy number using quantitative PCR (supplemental Figure S1). A phase resembling neutrophilic leukemia was observed in this patient 3 months before transformation to AML (WBC 159 x 10^9/L, with 75% neutrophils, 6% metamyelocytes and 14% myelocytes, but no blasts). At this stage, the neutrophils were already negative for JAK2-V617F (not shown). At transformation to AML, the purified granulocytes and leukemic blast cells isolated by FACS were negative for JAK2-V617F and displayed no 9pLOH (Figure 1B). Thus, the AML clone did not arise from a MPD progenitor heterozygous for JAK2-V617F that lost the mutated JAK2 allele during mitotic recombination. A similar conclusion was reached in another MPD patient who transformed to AML. Using the MPP1 expression assay to determine clonality by XCIP, we found that the granulocytes at MPD diagnosis were clonal (Figure 1A). At diagnosis of AML, peripheral blood granulocytes and leukemic blast cells purified from peripheral blood by FACS were clonal and expressed MPP1 from the same parental X-chromosome as the granulocytes at MPD diagnosis (Figure 1B). This result is compatible with a common origin of the MPD and AML clone, but does not rule out that two clones arose independently, since the AML blasts could fortuitously have inactivated the same X-chromosome as the MPD clone. In a second patient (NA-48) with initially JAK2-V617F positive MPD, we found at the time of leukemic transformation JAK2-V617F to be absent in isolated blast cells, but present in unfractionated bone marrow cells with an allelic ratio of 51%T (Table 1 and supplemental Figure S2). Del(11q) was found in 20/20 metaphases by cytogenetic analysis and in 99/100 cells by interphase FISH. This data suggests that the JAK2-V617F negative blasts and the JAK2-V617F positive bone marrow cells carry the same deletion, implying that they share a common clonal origin. Interestingly, the same region on chromosome 11 was previously identified in a genome-wide screening for LOH.
In summary, the unexpected finding of JAK2-V617F negative leukemia in patients with previously JAK2-V617F positive MPD could be explained by two models: first, MPD and AML represent two independent clones that arose de novo from different progenitor or stem cells, or second, MPD and AML are two subclones derived from a common ancestor. Our data on patient NA-02 are compatible with both models, whereas the results from patient NA-48 favor a common clonal origin of MPD and AML and suggest that an additional clonal event may precede the acquisition of JAK2-V617F in the pathogenesis of MPD.

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Authorship Contribution Statement:

A. Theocharides performed research, analyzed data and wrote the paper, M. Boissinot performed research and analyzed data, F. Girodon, R. Garand and E. Lippert provided
essential reagents and analyzed data; S.S. Teo performed research, P. Talmant performed cytogenetics and FISH studies, A. Tichelli analyzed data, S. Hermouet designed and performed research, analyzed data; R.C. Skoda designed research, analyzed data and wrote the paper.
Figure Legends

Figure 1. Analysis of clonal markers in patient NA-02.
A) Bone marrow samples at MPD diagnosis. The presence of the JAK2-V617F mutation was determined by allele-specific PCR (AS-PCR) using DNA from purified cells: CD15+, myeloid cells isolated by FACS; CD3+, T-cells isolated by FACS. %T indicates the allelic ratio between the mutant and wild-type JAK2 allele. Loss of heterozygosity on chromosome 9p (9pLOH) was determined with the microsatellite markers D9S1810 and D9S288 using DNA from purified cells as for JAK2. X-chromosome inactivation pattern (XCIP) was determined by allele-specific PCR for a G/T polymorphism in the MPP1 mRNA. The relative expression of the two MPP1 alleles was determined by comparing the G and T peak intensities obtained by the allele-specific RT-PCR assay. B) Peripheral blood samples at AML transformation. GRA, granulocytes isolated by Ficoll density centrifugation; CD34+, leukemic blasts isolated by FACS; CD3+, T-cells isolated by FACS. The assays were performed as in A).
References

A  Samples from patient NA-02 at MPD diagnosis

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B  Samples from patient NA-02 at AML transformation

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Table 1. Summary of patient characteristics and molecular analyses in AML secondary to MPD

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**Notes:**
1: %T determined in PB GRA.
2: %T determined in BM CD15+ cells.
3: sample was taken 9 months before transformation to AML (not at MPD diagnosis), but no signs of AML were present in PB at this time.
Leukemic blasts in transformed JAK2-V617F positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation

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