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

Alexandre Theocharides[1,2], Marjorie Boissinot[3], François Girodon[4], Richard Garand[5], Soon-Siong Teo[1], Eric Lippert[6], Pascaline Talmant[5], Andre Tichelli[7], Sylvie Hermouet[3,5] and Radek C. Skoda[1]

[1]Department of Research, Experimental Hematology, University Hospital Basel, Basel, Switzerland; [2]Division of Clinical Hematology, University Hospital Basel, Basel, Switzerland; [3]INSERM U601, Institut de Biologie, Nantes, France; [4]Laboratoire d'Hématologie, CHU de Dijon, Dijon, France; [5]Laboratoire d'Hématologie, CHU de Nantes, Nantes, France; [6]Laboratoire d'Hématologie, CHU de Bordeaux, Bordeaux, France, [7]Division of Diagnostic Hematology, University Hospital Basel, Basel, Switzerland

Reprints: Radek C. Skoda, MD, Department of Research, Experimental Hematology, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland, radek.skoda@unibas.ch

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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.

radek.skoda@unibas.ch

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.[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 thrombocythemia (ET) and >50% of idiopathic myelofibrosis (IMF).[2,5,6] Transformation to AML is a known complication of MPDs.[7-9] The frequency of *JAK2-V617F* in patients with de novo AML was around 1%.[10-15] In contrast, the *JAK2-V617F* mutation in AML secondary to MPD was in the range of 50%.[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.[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).[18-20]

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.[6,21,22]

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.[4] A quantitative PCR assay for *JAK2-V617F* was performed with two different methods,[6,22] 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.[22]

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-chromosomal 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.[23] Using the *MPP1* expression assay to determine clonality by XCIP,[24,25] 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.[26]

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 densitiy centrifugation; CD34+, leukemic blasts isolated by FACS; CD3+, T-cells isolated by FACS. The assays were performed as in A).

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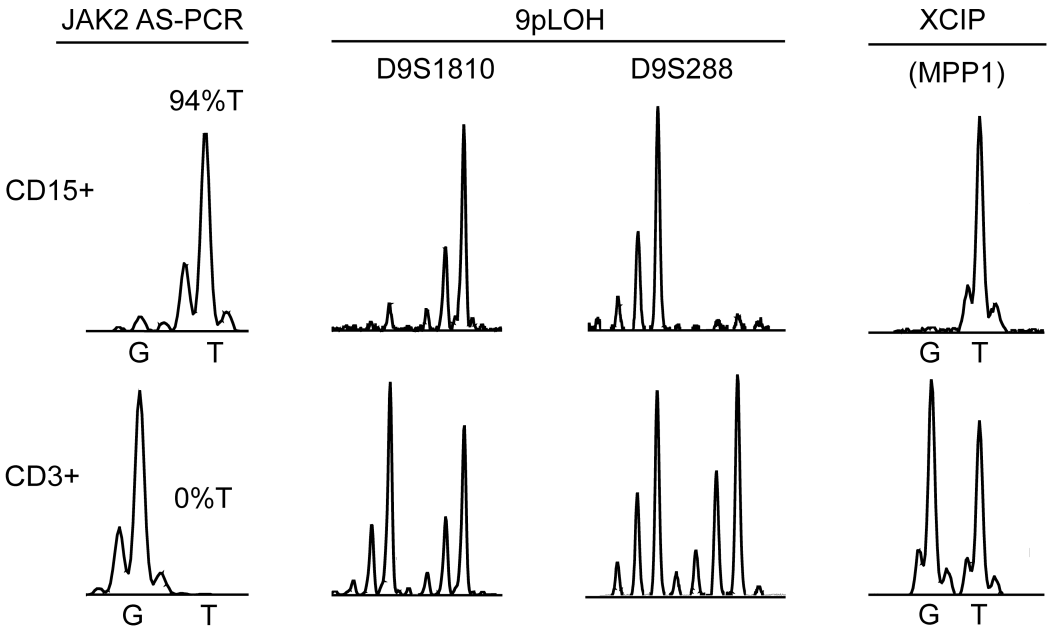
A Samples from patient NA-02 at MPD diagnosis



B Samples from patient NA-02 at AML transformation

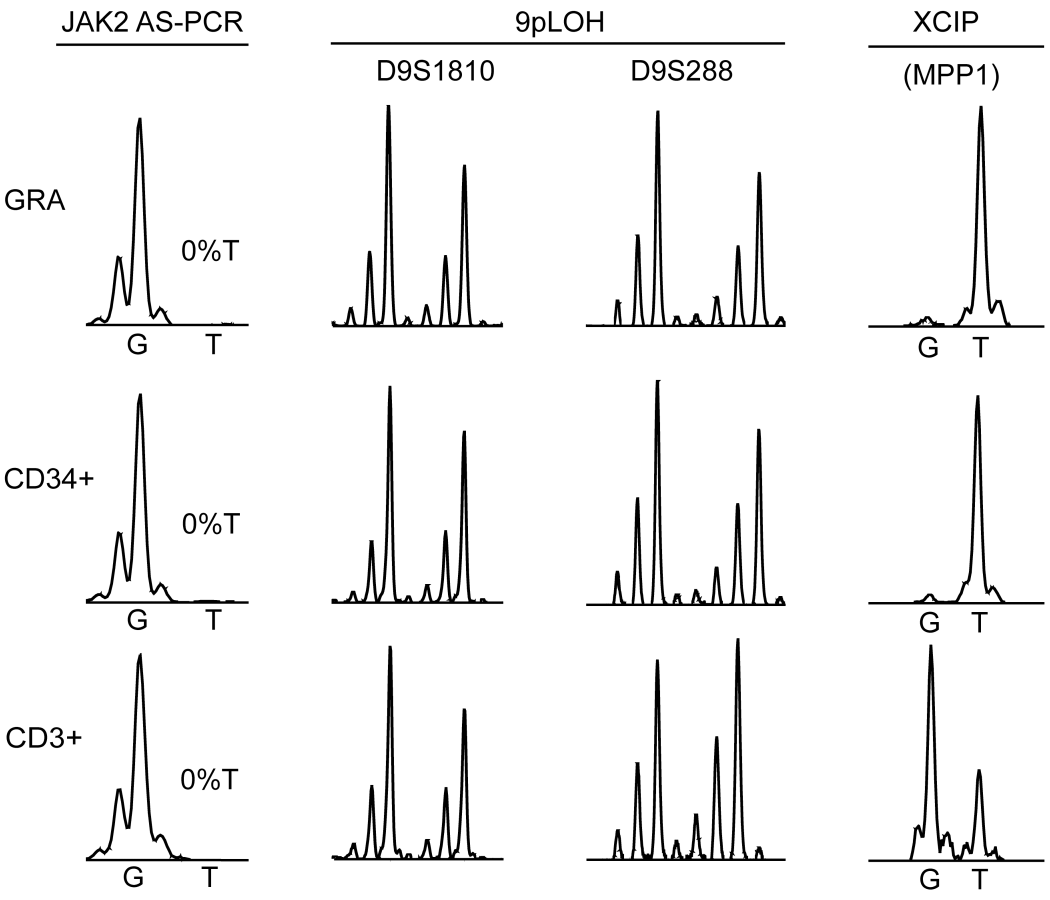


Table 1. Summary of patient characteristics and molecular analyses in AML secondary to MPD

		MPD						AML																	
								patient characteristics				unfractionated cell samples					purified cells								
														Nantes	Basel		granulocytic lineage			purified leukemic blasts			T-cells		
UPN	sex	Dx	age years	Nantes %T	Basel %T	source	cytoreductive treatment	FAB	age years	delay years	cytogenetics molecular abnormalities	%blasts	source	%T	%T	source	%T	source	method	%T	source	method	%T	source	method
V617F-positive MPD/ V617F-negative AML																									
NA-02	F	PV	70	79 [1]	94 [2]	GRA/BM-CD15+	Pipo	M2	72	2	normal	10	BM smear	0	0	BMMC	0	PB-GRA	Ficoll	0	PB-CD34+	FACS	0	PB-CD3+	FACS
NA-32	F	PV	70	42	52	PB GRA	Pipo	M1	72	2	nd	70	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
p128	M	PV	75	nd	30	PB GRA	HU	M4	80	5	Flt-3-ITD	58	PB smear	nd	0	PBMC	0	PB-CD15+	MACS	0	PB-CD34+	MACS	na	na	na
NA-01	M	PV	64	7	12	BM smear	Pipo	M0	72	8	nd	91	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
DI-03	M	ET	89	38	24	PB GRA	HU	na	91	2	-Y	42	PB smear	0	0	PBMC	0	PB-GRA	Ficoll	0	PB-CD34+	FACS	0	PB-CD3+	FACS
BO-04	F	ET	52	21	nd	PB GRA	none	M1	54	2	t(10;16)	93	PB smear	0	0	PBMC	0	PB-GRA	Ficoll	na	na	na	na	na	na
DI-05	M	ET	69	12	nd	BM smear	HU	M6	71	2	complex, del(5q), +7, -Y	52	BM smear	0	0	BMMC	0	BM-CD15+	FACS	0	BM-CD34+	FACS	na	na	na
DI-35	F	ET	68	30	53	BM smear	HU	M4	72	4	complex, del(5q)	45	BM smear	4	2	BMMC	0	BM-CD15+	FACS	0	PB/BM-CD34+	FACS	0	PB/BM-CD3+	FACS
NA-48	M	IMF	57	31	56	BM smear	HU/Thal	M2	59	2	complex, del(11q23)	35	BM smear	nd	51	BM smear	na	na	na	0	LC-blasts	LC	na	na	na
V617F-positive MPD/ V617F-positive AML																									
NA-59	M	PV	64	46	nd	PB GRA	HU/Pipo	na	67	3	nd	15	PB smear	75	nd	PBMC	90	PB-GRA	Ficoll	85	PB-CD34+	FACS	10	PB-CD3+	FACS
p185	M	PV	65	nd	30	BM smear	HU	M2	83	18	+8	75	BM smear	nd	69	PB-CD34+	65	PB-CD15+	FACS	69	PB-CD34+	FACS	na	na	na
NA-57	F	ET	47	82	89	PB GRA [3]	HU	na	60	13	complex, del(20q) , del(5q)	15	BM smear	71	80	PBMC	78	PB-GRA	Ficoll	100	PB-CD34+	FACS	0	PB-CD3+	FACS
DI-58	F	ET	64	26	34	BM smear	na	M2	78	14	complex, del(20q), -5, -7, +8	30	BM smear	41	66	BMMC	46	PB-GRA	Ficoll	56	PB-CD34+	FACS	1	PB-CD3+	FACS
DI-60	M	IMF	71	75	nd	BM smear	none	na	74	3	nd	73	PB smear	80	nd	PBMC	na	na	na	na	na	na	na	na	na
V617F-positive MPD/ V617F-uninformative AML																									
NA-30	M	PV	61	44	nd	BM smear	^{32}P/Pipo	M2	68	7	nd	18	BM smear	43	67	BM smear	na	na	na	na	na	na	na	na	na
DI-33	F	ET	63	16	nd	BM smear	HU/Pipo	M1	74	11	nd	91	BM smear	15	nd	BM smear	na	na	na	na	na	na	na	na	na
NA-52	M	IMF	64	44	40	BM smear	HU	M7	69	5	nd	24	BM smear	nd	59	BM smear	na	na	na	na	na	na	na	na	na
V617F-negative MPD/ V617F-negative AML																									
NA-07	M	PV	53	0	nd	BM smear	Pipo	M6	64	11	nd	75	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
NA-10	M	ET	67	0	0	BM smear	HU	M4	74	7	nd	65	PB smear	nd	0	PB smear	na	na	na	na	na	na	na	na	na
NA-11	F	ET	57	0	0	BM smear	HU/^{32}P/IFN/Ana	M2	73	16	nd	46	PB smear	nd	0	PB smear	na	na	na	na	na	na	na	na	na
DI-12	M	ET	47	0	nd	BM smear	HU	M7	49	2	complex	34	BM smear	0	nd	BM smear	na	na	na	na	na	na	na	na	na
DI-13	F	ET	81	0	0	BM smear	HU	M7	83	2	complex, del(5q),-7	36	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
DI-14	F	ET	75	0	0	BM smear	HU	M2	77	2	nd	43	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
NA-08	F	IMF	62	0	0	BM smear	HU/VP-16	na	69	7	nd	48	PB smear	nd	0	PB smear	na	na	na	na	na	na	na	na	na
NA-09	F	IMF	62	0	0	BM smear	Pipo/IFN	bi	73	11	nd	38	BM smear	0	0	BM smear	na	na	na	na	na	na	na	na	na
DI-20	M	IMF	65	0	nd	BM smear	na	M7	67	2	nd	14	BM smear	0	0	BMMC	na	na	na	na	na	na	na	na	na
p202	M	IMF	75	nd	0	BM smear	HU	na	80	5	nd	95	PB smear	nd	0	PBMC	na	na	na	na	na	na	na	na	na

UPN, unique patient number; M, male; F, female; Dx, diagnosis; nd, not done; na, not available; %T, percentage of chromosomes 9 carrying JAK2-V617F determined by allele-specific PCR; Nantes, allele-specific PCR was done in Nantes; Basel, allele-specific PCR was done in Basel; PB, peripheral blood; BM, bone marrow; GRA, granulocytes; PBMC, peripheral blood mononuclear cells; BMMC, bone marrow mononuclear cells; Pipo, pipobroman; HU, hydroxyurea; Thal, thalidomide; 32P, phosphorus 32, VP-16, etoposide, IFN, interferon alpha, Ana, anagrelide; none, no cytoreductive treatment; FAB, AML diagnosis according to FAB classification; bi, biphenotypic leukemia; delay, interval between MPD and AML diagnosis in years; %blasts, percentage of leukemic blasts on smear; LC-blasts, leukemic blasts isolated by laser capture microdissection.

Footnotes:

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.

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