Frequent reduction or absence of detection of the *JAK2*-mutated clone in *JAK2*V617F-positive patients within the first years of hydroxyurea therapy

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ABSTRACT

We analyzed the effect of hydroxyurea on the *JAK2V617F* allelic ratio (%*JAK2*V617F), measured in purified blood granulocytes, of patients with polycythemia vera and essential thrombocythemia. Thirty-six patients were examined sequentially prior to and after start of hydroxyurea therapy (8 polycythemia vera, 17 essential thrombocythemia), or while remaining untreated (2 polycythemia vera, 9 essential thrombocythemia). Hydroxyurea therapy (median duration: 15 months) reduced the %*JAK2*V617F by >30% in 13/25 patients (4 polycythemia vera, 9 essential thrombocythemia). For 3 patients, *JAK2*V617F remained undetectable for 3-27 months. In addition, a single time point study of two large cohorts of patients, examined either at the time of diagnosis (99 polycythemia vera, 178 essential thrombocythemia) or while receiving hydroxyurea (36 polycythemia vera, 98 essential thrombocythemia; median length of therapy: 32 months), confirmed reduction of %*JAK2*V617F in the hydroxyurea-treated group (24% *vs.* 33% *JAK2*V617F at diagnosis, $p<0.01$). Prospective studies are needed to determine the prognostic value of reduced *JAK2*V617F allele burden under cytoreductive therapy.

Key words: *JAK2*V617F, hydroxyurea, myeloproliferative neoplasm, polycythemia vera, essential thrombocythemia, allele-specific real time quantitative PCR.

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Introduction

Detection of the V617F mutation of *JAK2* (*JAK2*V617F), recently discovered as specifically expressed in the majority of BCR-ABL-negative myeloproliferative diseases (MPDs), has become a main diagnostic test for polycythemia vera (PV) and essential thrombocythemia (ET).[1-3] In contrast, the relevance of the *JAK2*V617F mutation load for predicting the risk of thrombosis, myelofibrosis or leukemic transformation remains controversial.[4-9] The lack of consensus may be explained by heterogeneity in techniques and materials (purified cells, whole blood, genomic DNA, cDNA) used to quantify *JAK2*V617F, and by the cohorts of patients studied. Indeed, since the *JAK2*V617F load is currently thought not to be affected by conventional treatments, most of the published studies of the *JAK2*V617F mutation load have mixed treated and untreated patients. However, the insensitivity of the *JAK2*V617F-mutated clone to conventional therapy has never been proven. So far the *JAK2*V617F allelic ratio (%*JAK2*V617F) has usually been analyzed at a single time point. Studies analyzing the %*JAK2*V617F sequentially are rare: Passamonti *et al.* described an increase in %*JAK2*V617F in a cohort of 8 patients over a mean period of 17 months.[10] Kiladjian *et al.* reported a high molecular response rate in PV patients treated with interferon α-2a (IFNα-2a).[11] Using sequential %*JAK2*V617F analyses, Barosi *et al.* described transformation from heterozygous to homozygous forms of primary myelofibrosis.[12] Surprisingly, the effect of hydroxyurea (HU), an inexpensive drug widely used in MPDs, on the *JAK2*V617F mutation load is still unknown. To address this question, we quantified *JAK2*V617F in purified blood granulocytes from three distinct cohorts of PV and ET patients. A first group of patients was examined at diagnosis then followed as they remained untreated (11 patients) or received HU (25 patients), then two large cohorts of patients, either at the time of diagnosis or under treatment with HU, were studied retrospectively.

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Design and Methods

Patients

Patients from two centers diagnosed with MPD according to the WHO criteria were studied as three groups. Thirty-six patients from center 1 (10 PV, 26 ET) were examined first at diagnosis then sequentially without (11 patients) or with HU treatment (25 patients). In a second, retrospective study, two distinct groups of patients were examined at a single time point: group A (n=277; 99 PV, 178 ET; 124 from center 1, 153 from center 2) was examined at the time of diagnosis; group B (n=134; 36 PV, 98 ET; 51 from center 1, 83 from center 2), while under treatment with HU. The study was approved by the institutional ethics committee on human experimentation, Comité de Protection des Personnes Est I and informed consent was provided according to the Declaration of Helsinki.

Analysis of %JAK2V617F

Purified blood granulocytes were prepared in the two centers as described.[13] Cytospins, stained with May-Grünwald-Giemsa, confirmed the purity (>95%) of the granulocyte preparations. Genomic DNA was prepared using QIAamp DNA mini-kits (Qiagen). In both centers, levels of expression of *JAK2* wild-type (WT) or mutated (V617F) were determined in duplicate using the same sensitive (0.15% *JAK2*V617F) allele-specific quantitative PCRs (AS-qPCR) with the specificity based on *sense* forward primers.[13] Copy numbers were determined by comparison with serial dilutions of plasmids.[13] Absence of significant inter-center variability in the AS-qPCR assay was verified by parallel analysis in both centers of 70 DNA samples (*data not shown*). To limit inter-assay variability, sequential samples from a same patient were analyzed in the same AS-qPCR run. Whenever the *JAK2*V617F allele burden decreased by >30% or became undetectable during treatment, quantification was repeated on the same sample, then on new samples. A second AS-qPCR assay with the specificity based on *anti-sense* reverse primers was developed to verify the *JAK2*V617F allelic ratio in HU-treated patients. Sequences of primers and probes were: forward primer, 5'-GCGCGTGCATCTTTATTATGGCAGA-3'; *JAK2* wild-type reverse primer, 5'-CCCGTTACTCTCGTCTCCACAGAC-3'; *JAK2*V617F reverse primer, 5'-GCCGTTACTCTCGTCTCCACAGAA-3'; and probe, 5' 6FAM-GAGAAAGC TTGCTCATCATACTTGCTGC-3' TAMRA.

Statistical analysis

Mean comparison one-sided parametrical test was used to compare different groups of patients, and Fisher's exact or χ^2 tests to compare proportions. Analysis of sequential %*JAK2*V617F was made with a t-test. Data were analyzed using the Stata software. $p<0.05$ was considered statistically significant.

Results and Discussion

The effect of HU on the *JAK2*V617F-mutated clone was studied with sequential assessment of %*JAK2*V617F in 36 patients. These patients (10 PV, 26 ET) had at least two determinations of the *JAK2*V617F mutation load, at the time of diagnosis and after a median period of 16 months of follow-up, with or without HU (Figure 1). Twenty-five patients were treated with HU (8 PV, 17 ET) with a 15-month median duration of treatment at the time of the second assessment of the %*JAK2*V617F; 11 patients (2 PV, 9

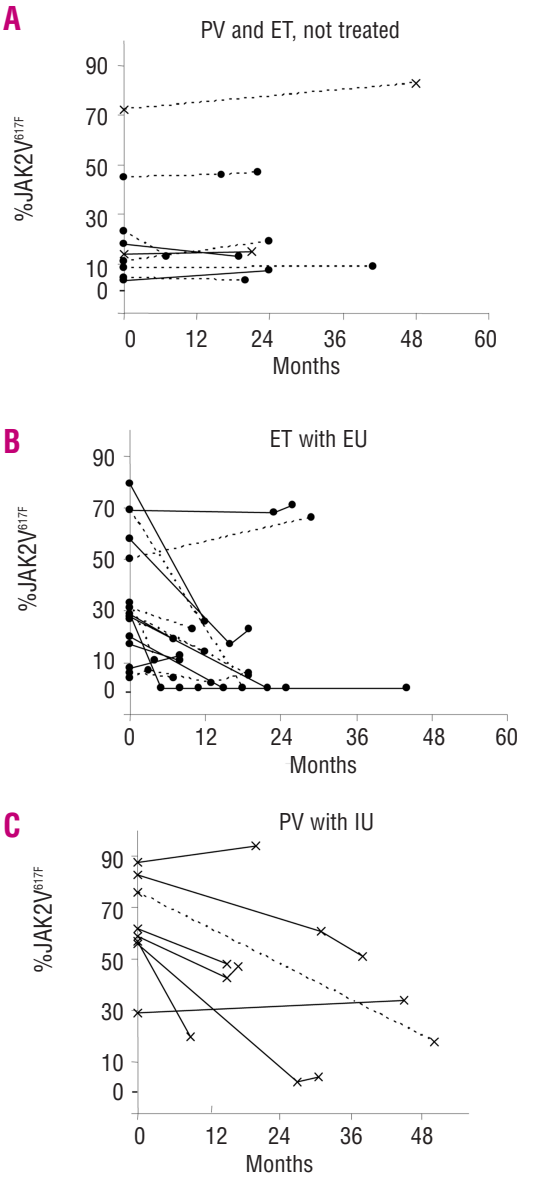

Figure 1. Evolution of the %*JAK2*V617F in treated and untreated polycythemia vera and essential thrombocythemia patients. (A) Patients who did not receive any cytoreductive treatment. (B) essential thrombocythemia patients who were treated with hydroxyurea for the indicated time (expressed in months). (C) polycythemia vera patients who were treated with hydroxyurea for the indicated time (expressed in months). There was no significant change in %*JAK2*V617F in untreated patients. The %*JAK2*V617F was significantly decreased for both essential thrombocythemia and polycythemia vera patients who received HU ($p<0.05$). (– –) female patients; (—) male patients.

ET) did not receive cytoreductive therapy (median follow-up: 16 months; range: 1-47 months). The group of ET patients to whom HU was prescribed had higher platelet counts at diagnosis than patients who were not given HU (758 *vs.* 620 G/L, p=0.0289). Consistent with aggressive forms of MPD justifying cytoreductive therapy, they also had a higher %*JAK2*V617F at diagnosis (PV, 64%; ET, 34%) than patients who remained untreated (PV, 43%; ET, 20%). In the group of untreated patients, the %*JAK2*V617F remained stable (Figure 1A). In contrast, treatment with HU resulted in decreased %*JAK2*V617F, from 43% at diagnosis to 24% during HU-treatment (p<0.0001). The decrease in %*JAK2*V617F was significant in PV (from 64 to 40%, p=0.0141) and in ET (from 34 to 16.7%, p<0.01) (Figure 1 B, C). Of note, a >30% decrease was noted in 13 patients (5 females, 8 males). *JAK2*V617F became undetectable in 4 ET patients (1 female, 3 males) after a 5-55 month period of treatment, and remained undetectable for a 3-27 month period of follow-up.

Table 1. %*JAK2*V617F analysis in patients tested before and during hydroxyurea treatment.

				%*JAK2V617F*					
				Before treatment		*During HU treatment*			
ID N.	*Dg.*	*Sex*	*Age**	*AS-q PCR 1*	*Duration***	*AS-q PCR 1*	*AS-q PCR 2*	*Mean*	*Variation*
Patients with molecular response >30%									
D690	ET	F	75 yrs	33%	4 mo.	11%	17%	14%	-58%
D469	ET	M	54 yrs	28%	5 mo.	0%	0%	0%	-100%
D525	PV	M	73 yrs	57%	9 mo.	20%	24%	22%	-61%
D490	ET	M	75 yrs	79%	12 mo.	26%	43%	35%	-56%
D335	ET	F	76 yrs	27%	12 mo.	14%	17%	15%	-44%
TD66	ET	M	66 yrs	20%	15 mo.	0%	0%	0%	-100%
D348	ET	M	51 yrs	58%	16 mo.	17%	15%	16%	-72%
D273	ET	F	32 yrs	69%	18 mo.	0%	0%	0%	-100%
D317	ET	F	29 yrs	29%	19 mo.	5%	7%	6%	-79%
D212	ET	M	78 yrs	28%	22 mo.	0%	1%	0.5%	-98%
D161	PV	M	44 yrs	56%	27 mo.	2%	4%	3%	-95%
D110	PV	M	68 yrs	83%	31 mo.	61%	56%	58%	-30%
D63	PV	F	55 yrs	76%	50 mo.	18%	13%	15%	-80%
Patients with no molecular response or response <30%									
D216	ET	M	71 yrs	4%	1 mo.	7%	9%	8%	+100%
D529	ET	F	87 yrs	6%	7 mo.	4%	8%	6%	0
D670	ET	M	83 yrs	29%	7 mo.	19%	23%	21%	-28%
D501	ET	M	78 yrs	14%	8 mo.	23%	33%	28%	+100%
D504	ET	M	71 yrs	17%	8 mo.	11%	18%	14%	-18%
D478	ET	F	94 yrs	31%	10 mo.	23%	24%	23%	-27%
D301	PV	M	77 yrs	59%	15 mo.	70%	79%	74%	+25%
D434	PV	M	72 yrs	62%	15 mo.	48%	62%	55%	-11%
D324	PV	M	70 yrs	88%	20 mo.	94%	92%	93%	+6%
D245	ET	M	65 yrs	69%	23 mo.	68%	76%	72%	+4%
TD132	ET	F	61 yrs	50%	29 mo.	66%	68%	67%	+34%
D80	ET	M	34 yrs	29%	45 mo.	34%	ND	ND	+17%

*Patients were tested first at the time of diagnosis then at variable time points after the start of HU therapy, using two AS-qPCR assays: AS-qPCR 1, with specificity based on forward, sense primers, and AS-qPCR 2, with specificity based on reverse, anti-sense primers. Data presented are mean values of duplicates or triplicates. ID N: patient identification number; Dg: diagnosis. *Age at the time of diagnosis; yrs: years. **Duration of treatment at time of JAK2V617F analysis: mo.: months. Mean: mean of %JAK2V617F results obtained with AS-qPCRs 1 and 2. ND: not done. Variation: [(mean JAK2V617F during HU treatment - %JAK2V617F at diagnosis)/(JAK2V617F at diagnosis)] x 100.*

Decreases in %*JAK2*V617F were confirmed by a second AS-qPCR assay (Table 1). Importantly, 3 of the 4 ET patients found negative for *JAK2*V617F while receiving HU were confirmed negative by the second AS-qPCR assay; the fourth patient was found positive, but with 1% *JAK2*V617F only.

The effect of HU on the %*JAK2*V617F was then investigated in a retrospective study of large cohorts of PV and ET patients, using two distinct groups of patients tested at a single time point. Patients of group A were examined at the time of diagnosis. *JAK2*V617F was not detected in granulocytes of 5/99 PV patients (5%) and 51/178 ET patients (29%) (Table 2). These cohorts of PV and ET patients were comparable to those previously described.[13] Mean %*JAK2*V617F in patients carrying the mutation was 54% in PV and 18% in ET. A minority of PV (13/99, 13%) had <25% *JAK2*V617F and a minority of ET (10/178, 6%) had >40% *JAK2*V617F. *JAK2*V617F -positive ET patients were older, with lower platelet counts and higher hematocrit and hemoglobin levels (controlled for gender) than *JAK2*V617F-negative ET patients. The proportion of male patients was 51% for PV and 40% for ET. There was no difference in *JAK2*V617F mutation load between male and female patients; this was true in both the PV and ET groups.

Patients of group B, a distinct cohort, had been treated with HU for a median duration of 32 months (same median duration of treatment for PV and ET patients), with no significant difference in treatment duration between male and female patients. The mean ages of PV and ET patients were similar in groups A and B; the proportions of male patients in group B were 50% for PV and 40% for ET. As expected, hematocrit, hemoglobin level, white blood cell (WBC) and platelet counts were significantly lower in the treated group (for both PV and ET) than in the group at diagnosis (Table 2). HU-treated patients had a significantly lower *JAK2*V617F allelic ratio (24%) than patients at diagnosis (33%, p<0.01). *JAK2*V617F was not detected in granulocytes from 5/36 treated PV patients (14%) and 34/98 treated ET patients (35%); these proportions were not significantly different from those of group A.

Interestingly, under HU treatment, PV patients with <25% *JAK2*V617F (15/36, 7 males, 8 females) represented a higher percentage than in the PV cohort at diagnosis (42% *vs.* 13%, p<0.001). Consistently, treated PV patients had a lower %*JAK2*V617F than PV patients at diagnosis (44% *vs.* 54%, p=0.0257). There was no difference in %*JAK2*V617F for the group of ET patients under treatment compared to those tested at time of diagnosis. However, analysis of ET data by gender revealed a lower %*JAK2*V617F during HU therapy for female patients (p<0.01). The same analysis of PV also showed that the decrease in %*JAK2*V617F associated with HU treatment was significant only in women (39% with HU *vs.* 53% at diagnosis, p=0.0249), suggesting that the *JAK2*V617F-mutated clone may be more sensitive to HU in female than in male MPD patients. A similar gender difference had previously been reported by Pemmaraju *et al.*[7] in a series of 80 ET patients that included treated patients.

Table 2. Hematologic and clinical features of patients.

	Group A: tested at the time of diagnosis				*Group B: tested while receiving HU*				*Comparison of Group B vs. Group A*
	All	*V617F+*	*V617F–*	*p*	*All*	*V617F+*	*V617F–*	*p*	*p**
ET									
Males/Females	70/108	50/77	20/31		40/58	25/39	15/19		
Age (mean±SD)	63±16	65±16	59±18	0.01	64±14	66±14	60±13	0.03	NS
Hematologic parameters (mean±SD)									
Hemoglobin	13.6±1.6	14.0±1.5	12.8±1.5	0.0001	12.9±2.1	13.0±2.2	12.7±1.9	NS	0.0002
Hematocrit	42.0±6.4	43.2±6.7	39.1±4.7	0.0001	39.9±6.2	40.1±6.6	39.5±5.6	NS	0.0016
MCV	90±5	90±6	90±4	NS	106±12	104±10	109±14	0.0276	<0.0001
WBC	10.0±4.0	10.1±4.1	9.7±3.1	NS	6.9±3.2	6.9±2.8	7.0±3.7	NS	<0.0001
platelets	819±368	753±234	981±552	0.0001	524±274	497±232	576±340	NS	<0.0001
% *JAK2*-V617F (mean±SD)									
All	13±13	18±12	0	NS	10±14	15± 15	0		NS
Males	14±13	19±12	0	males *vs.*	11±16	18± 16	0	males *vs.*	NS
Females	12±13	17±12	0	females	8±13	13±14	0	females	0.0364
PV									
Males/Females	51/48	48/46	3/2		18/18	15/16	3/2		
Age (mean±SD)	71±11	72±10	56±17	0.0007	67±14	68±13	63±20	NS	NS
Hematologic parameters (mean±SD)									
Hemoglobin	18.7±1.7	18.7±1.7	18.8±0.5	NS	14.7±2.7	14.8±2.8	14.3±2.2	NS	<0.0001
Hematocrit	58.2±4.9	58.2±5.0	57.9±2.4	NS	45.2±9.2	45.4±9.5	44.2±7.6	NS	<0.0001
MCV	86±8	86±8	88±7	NS	103±12	104±12	98±8	NS	<0.0001
WBC	12.6±5.0	12.8±5.1	8.1±2.0	0.0202	10.4±9.6	10.6±10.0	9.1±6.7	NS	0.0495
Platelets	433±200	433±200	432±232	NS	336±147	314±128	477±189	0.0093	0.0011
% *JAK2*-V617F (mean±SD)									
All	51±27	54±24	0	NS	38±31	44±29	0	NS	0.0257
Males	52±29	56±27	0	males *vs.*	41±33	49±30	0	males *vs.*	NS
Females	50±24	53±22	0	females	35±28	39±27	0	females	0.0249

Groups A and B were distinct groups of patients. Group A was tested at a single time point, at the time of diagnosis (before receiving any treatment). Group B was tested at a single time point while already receiving HU therapy (median duration of treatment: 32 months). p: comparison of group A ("V617F+") and group B ("V617F+"). NS: not statistically significant. MCV: mean corpuscular volume; WBC: white blood counts.*

The finding that HU, the drug most commonly used in MPDs, reduces and occasionally renders the *JAK2*V617F-mutated clone undetectable with sensitive (0.15%) AS-qPCR assays underlines the importance of detecting and quantifying *JAK2*V617F before initiating cytoreductive therapy. This is necessary in order to rule out false negatives under treatment. In this regard, *JAK2*V617F-negative should be applied solely to patients for whom the V617F mutation was not detectable prior to treatment. Furthermore, molecular response to HU, in addition to assay sensitivity, may explain the differences in ratios of *JAK2*V617F-positivity for ET reported in studies mixing treated and untreated patients (usually 50% *JAK2*V617F-positive) compared to studies of patients at diagnosis (70-75% *JAK2*V617F-positive).[7,8,13,14]

The delay and length of response of the JAK2-mutated clone to HU therapy could not be evaluated in this mostly retrospective study. However, all but 2 patients who responded to HU had been treated for <3 years (median: 18 months) and decreases in *JAK2*V617F allele burden were observed in 2 patients with <6 months of treatment, suggesting that reduction of the *JAK2*V617F -mutated clone likely occurs within the first year of HU therapy. This could explain the apparent contradictions between our study and others, which reported no change in %*JAK2*V617F in sequential analysis of treated MPD patients, likely under HU-therapy for several years when first tested for *JAK2*V617F.[15] We tried to compare patients with molecular response to HU with those who did not respond. The only difference between ET patients responding to HU with decreased %*JAK2*V617F and those with no variation was younger age (mean age: 60 *vs.* 71 years respectively, $p<0.05$). There was no difference in blood parameters (WBC, platelet counts, Ht, Hb) at the time of diagnosis. Similarly, blood counts of patients for whom *JAK2*V617F became undetectable with HU treatment did not differ from those with moderate or no decrease in %*JAK2*V617F.

Finally, it is important to determine whether one should aim towards complete molecular response in *JAK2*V617F-positive patients. As treatment affects the (mutated) granulocyte/(non-mutated) lymphocyte ratio, accurate assessment of the molecular response of treated patients – quantification of low levels of *JAK2*V617F – can only be achieved using purified granulocytes, not whole blood.[16] However, the prognostic

value of the *disappearance* of the *JAK2*V617F-mutated clone during treatment is currently not known. We and others previously reported sharp decreases and disappearance of *JAK2*V617F-mutated clones in PV and ET patients in association with transformation into acute myeloid leukemia.[17] In the present series, none of the patients who became *JAK2*V617F-negative with HU therapy showed signs of leukemic transformation for as long as 27 months. Prospective studies with sequential assessments of %*JAK2*V617F – taking into account gender – are needed to determine whether obtaining a molecular response has a favorable influence on MPD evolution, compared to patients for whom the *JAK2*V617F load remains stable.

Authorship and Disclosures

FG and SH conceived the study, analyzed the data, wrote the paper and created Table 2; CC performed AS-qPCRs; MM carried out statistical analyses, CS collected and analyzed the data, created Table 1 and Figure 1; FDS and AV collected the data; IL enrolled patients, MM enrolled patients and revised the manuscript, FG is responsible for the patient database.

All the individuals listed as co-authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship as detailed in the ICMJE website: http://www.icmje.org/#author. The authors reported no potential conflicts of interest.

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