Clinical and biological characterization of patients with low (0.1-2%) *JAK2*V617F allele burden at diagnosis

Detection of the *JAK2*V617F mutation is of major help in the diagnosis of myeloproliferative neoplasms (MPNs).[1-3] Techniques using allele-specific quantitative PCR (AS-qPCR) can reliably and consistently detect down to 0.001% mutated alleles.[4] Moreover, a study of healthy blood donors has shown that the maximum *JAK2*V617F value in 200 subjects was 0.035%.[5] In practice, a positivity threshold of 1% is commonly accepted.[6] The generalization of highly sensitive techniques revealed that the detection of very small clones (around and below 1% *JAK2*V617F) is far from exceptional and the interpretation of this is sometimes challenging. In particular, one may question the clinical interest in detecting such minor clones in untreated patients. Molecular biologists and clinicians involved in MPN management in the Myeloproliferative Neoplasms and Related Disorders - European Network (MPN&MPNr-EuroNet) have collected and analyzed clinical and biological data from 36 patients presenting with 0.1-2% *JAK2*V617F at diagnosis.

All patients had provided an informed written consent in accordance with the Declaration of Helsinki for the use of remnant DNA for investigational purposes. Local ethics committees approved the study. Eight molecular biology laboratories members of the MPN&MPNr-EuroNet were sent DNA standards containing 0.1 % and 1% JAK2V617F prepared and provided by IPSOGEN (now Qiagen-Marseille, France) to calibrate local assays. With the help of these standards, 36 patients were selected who fulfilled the following criteria: *JAK2*V617F determined at least twice with at least two months between the two determinations; one determination found between 0.1% and 2% *JAK2*V617F in the absence of cytotoxic therapy; sufficient clinical and biological characterization to confirm or rule out the diagnosis of MPN; sufficient DNA for centralized assessment of *JAK2*V617F allele burden and search for additional mutations. Each center first used their own *JAK2*V617F quantification technique and sent DNA to the CHU de Bordeaux where the allele burden was centrally determined using a "sense" and an "anti-sense" primer-based allele-specific qPCR as described[3,7] (Figure 1). For patients with erythrocytosis, *JAK2* exon 12 mutations were screened by high resolution melting curve analysis (HRM)[8] followed by sequencing. For patients with thrombocytosis or suspicion of myelofibrosis, *MPL* exon 10 and *CALR* mutations were detected by HRM or fragment analysis and confirmed by sequencing.

Patients were subdivided into four groups depending on the reason for which they underwent *JAK2*V617F mutation detection: erythrocytosis (n=15), unusual thrombosis (n=3), thrombocytosis (n=11) or suspicion of myelofibrosis (circulating immature granulocytes, anemia and/or splenomegaly; n=7). The correlation between the allele burdens determined locally and in the central laboratory was not perfect, a few results being under-estimated in local laboratories (Table 1). However, all positive results were confirmed positive by central determination. This underlines the technical difficulty of precisely assessing low burdens and the usefulness of centralized assessment of low *JAK2*V617F quantification results.

Among the 15 patients explored for erythrocytosis, only 3 had a final diagnosis of polycythemia vera (PV) according to WHO criteria (omitting the *JAK2*V617F mutation). Of these, one (E4) had an additional mutation in exon 14, just upstream (5') of the G1849T (V617F) substitution, thus interfering with the annealing of the primer and resulting in an underestimation of the *JAK2*V617F burden when assessed with a "sense" qPCR. These cases can be detected by using an "anti-sense" qPCR with the specific primer located 3' of the mutation (Figure 1). For patient E4, the alternative PCR revealed a significant mutated clone (10% *JAK2*V617F). The same phenomenon applies to patient M7; in this case, the "sense" qPCR indicated a *JAK2*V617F burden of 0.6% while the “anti-sense” revealed a high burden (73%). Thus, an unexpectedly low *JAK2*V617F burden should first prompt a search for additional mutations that might be hampering correct primer annealing. Another PV patient (E8) had a mutation in the exon 12 of *JAK2* in addition to the small (1.5%) *JAK2*V617F clone. The third patient with a PV phenotype (E14) did not fulfill WHO criteria of PV at the time of diagnosis because he had a normal EPO level and bone marrow histology was not consistent with PV. However, when *JAK2*V617F was later reassessed, it was found to have slightly increased levels (from 0.6% to 1.5%) and the EPO level was then found below the lower limit. Thus, in this case, enough criteria were gathered to establish the diagnosis of PV based on the WHO recommendations. In the 12 remaining patients with erythrocytosis, none had sufficient criteria to confirm the diagnosis of PV. Three (patients E1, E2 and E10) had secondary erythrocytosis attributed to a respiratory condition (chronic obstructive pulmonary disease). Three others (patients E3, E9 and E13) had probable secondary erythrocytosis with high levels of EPO. Two (patients E11 and E15) had a false erythrocytosis diagnosed on the basis of a normal isotopic red cell mass, and one (patient E12) had a transitory erythrocytosis that resolved spontaneously. Three others (patients E5, E6 and E7) had idiopathic erythrocytosis of whom 2 remained stable without treatment. Two of these 3 patients did not have a bone marrow biopsy, so a diagnosis of MPN could not be definitively ruled out. None of the patients explored for erythrocytosis evolved towards myelofibrosis or leukemia.

For the 3 patients tested for unusual thromboses (which did not include splanchnic thromboses), no sign of MPN was found and blood counts remained normal.

For the 11 patients presenting with thrombocytosis, a clear diagnosis of MPN could be made in 5, either because the bone marrow histology was in favor of MPN, and/or because an additional mutation was found in the MPL (Tc3) or *CALR* (Tc5 and 11) genes. For the other patients, even though the bone marrow histology was not available (Tc6 and Tc9), or was reported as normal (Tc1, Tc2, Tc7 and Tc8), the persistence of high platelet counts in the absence of any reactive cause of thrombocytosis made the diagnosis of essential thrombocythemia (ET) highly probable. For patients tested for suspicion of myelofibrosis, a hematologic malignancy was always confirmed: 3 primary myelofibrosis (PMF), 3 myelodysplastic syndromes, and one overlapping syndrome (atypical chronic myeloid leukemia). The evolution of these patients was mostly pejorative.

These results indicate that when a low *JAK2*V617F burden is found, one should first eliminate the possibility that the %*JAK2*V617F is under-estimated because of an additional mutation hampering correct primer or probe annealing. Secondly, the existence of another mutation should be searched for, either in *JAK2* exon 12 for patients with erythrocytosis or in the *CALR* or *MPL* genes for other

Table 1. Main characteristics of 36 patients with a low JAK2V617F allele burden.

Patient n.	%*JAK2*V617F (local measurement)	%*JAK2*V617F (centralized)	Bone marrow histology	EPO (mUI/mL)	EEC/EMC	Other *JAK2* mutation	MPL mutation	CALR mutation	Final diagnosis
E1	0.39	1.26	Erythroblastic hyperplasia	11.8	ND	None	ND	Neg	Pulmonary Disease
E2	0.70	0.91	Normal	3.4	ND	None	ND	Neg	Pulmonary Disease
E3	0.70	2.99	Normal	13.1	ND	None	ND	Neg	Secondary Erythrocytosis
E4	0.20	10.00	PV	ND	Pos/Pos	V615L	ND	Neg	Idiopathic Erythrocytosis
E5	1.40	1.40	ND	ND	Neg/Neg	None	ND	Neg	Idiopathic Erythrocytosis
E6	1.84	1.81	ND	3	Neg/Neg	None	ND	Neg	Idiopathic Erythrocytosis
E7	1.00	1.00	Normal	7.9	Neg/Neg	None	ND	Neg	Idiopathic Erythrocytosis
E8	1.50	1.50	ND	ND	Pos/Neg	F537I K539I	ND	Neg	Idiopathic Erythrocytosis
E9	0.70	0.40	ND	27.2	ND	None	ND	Neg	Secondary Erythrocytosis
E10	0.70	0.54	ND	11.6	ND	None	ND	Neg	Pulmonary Disease
E11	0.20	0.20	ND	ND	ND	None	ND	Neg	False Erythrocytosis
E12	0.40	0.40	No MPN	2.48	Neg/Neg	None	ND	Neg	Transitory Erythrocytosis
E13	0.45	0.90	Erythroblastic hyperplasia	16.7	Pos/Neg	None	ND	Neg	Secondary Erythrocytosis
E14	0.58	0.77	Normal	5.6	Pos/Neg	None	ND	Neg	Idiopathic Erythrocytosis
E15	1.00	1.00	ND	10	ND	None	ND	Neg	False Erythrocytosis
Ts1	0.84	1.07	Normal	ND	ND	ND	ND	Neg	No MPN
Ts2	0.50	2.87	ND	13	Neg/Neg	ND	ND	Neg	No MPN
Ts3	0.45	0.45	Normal	ND	ND	ND	ND	Neg	No MPN
Tc1	0.70	4.64	Normal	ND	ND	ND	Neg	Neg	Probable ET
Tc2	0.50	1.33	Normal	10.9	ND	ND	Neg	Neg	Probable ET
Tc3	0.27	0.28	ET	ND	ND	ND	W515L	Neg	ET
Tc4	1.38	1.20	ET	ND	ND	ND	Neg	Neg	ET
Tc5	0.10	0.30	ND	ND	Neg/Pos	ND	Neg	Type 1 mutation	ET
Tc6	0.70	1.40	ND	6	ND	ND	Neg	Neg	Probable ET
Tc7	1.66	5.78	Normal	ND	Pos/Pos	ND	Neg	Neg	Probable ET
Tc8	2.00	3.36	Normal	ND	Neg/Neg	ND	Neg	Neg	Probable ET
Tc9	0.60	1.50	ND	ND	ND	ND	Neg	Neg	Probable ET
Tc10	0.50	0.50	Primary Myelofibrosis	ND	ND	ND	Neg	Neg	Primary Myelofibrosis
Tc11	1.00	0.42	ND	6.76	ND	ND	Neg	Type 1 mutation	ET
M1	0.80	1.18	atypical CML	ND	ND	ND	Neg	Neg	Atypical CML
M2	0.74	0.64	mds/mpn	9.49	ND	ND	Neg	Neg	Myelodysplasia Myelofibrosis
M3	0.69	0.93	Primary Myelofibrosis	ND	ND	ND	Neg	Neg	Primary Myelofibrosis
M4	0.93		MDS	ND	ND	ND	Neg	Neg	MDS
M5	2.00	0.80	MDS	ND	ND	ND	Neg	Neg	MDS
M6	0.80	0.80	Primary Myelofibrosis	ND	ND	ND	Neg	Neg	Primary Myelofibrosis
M7	0.60	73.00	Primary Myelofibrosis	ND	ND	c.1848_1849delinsCT	Neg	Neg	Primary Myelofibrosis

EPO: erythropoietin; EEC: endogenous erythroid colonies; EMC: endogenous megakaryocytic colonies; PV: polycythemia vera; ET: essential thrombocythemia; MDS/MPN: mixed myelodysplastic/myeloproliferative neoplasm; ND: no data available.

patients. These associations are rare but other cases have been reported[9,10] and their finding is consistent with the observation that mutations which activate cytokine signaling pathways can be acquired several times in MPNs. In particular, the *JAK2*V617F mutation itself has been shown to be acquired at least twice in at least 2.8% of MPN patients.[11]

In the absence of other mutations in the *JAK2*, *CALR* or *MPL* genes, the interpretation varies according to the context. For patients with erythrocytosis, the diagnosis of PV cannot be established based on low *JAK2*V617F allele burdens since most of the patients in our cohort showed no evidence of PV and some had clear evidence of secondary erythrocytosis. In this context, the presence of a minor clone with acquired *JAK2*V617F is reminiscent of the mosaicisms of cancer-associated mutations found in

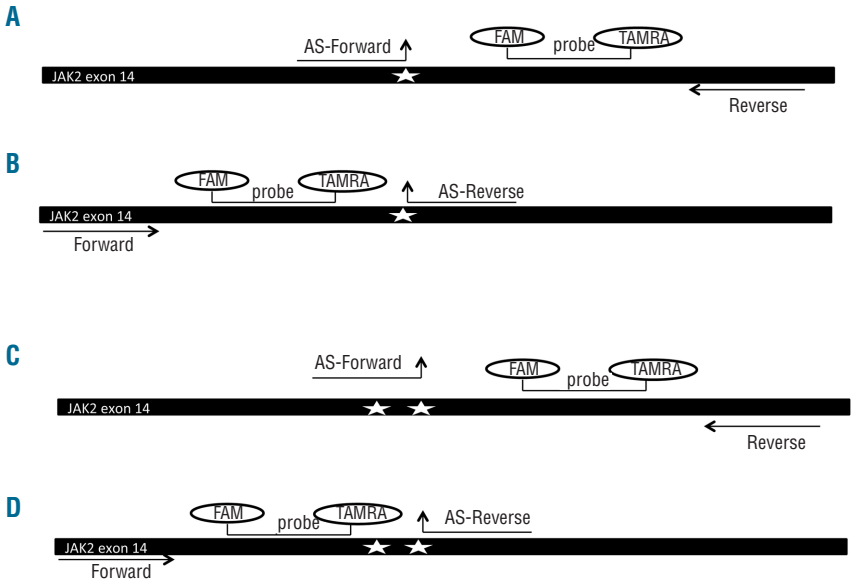

Figure 1. Design of the «sense» and «anti-sense» *JAK2*V617F allele-spectific qPCR. (A) In the «sense» PCR, the forward primer is allele-specific whereas the reverse primer and the FAM-TAMRA probe are generic. (B) In the antisense-PCR, allele specificity is based on the reverse primer. (C) In the case of an additional mutation occuring 5' of the G1849T substitution, annealing of the forward allele-specific primer is impaired with the «sense» PCR, resulting in an under-estimation of the mutant allele burden. (D) In the case of an additional mutation occurring 5' of the G1849T substitution, the «anti-sense» PCR is not affected.

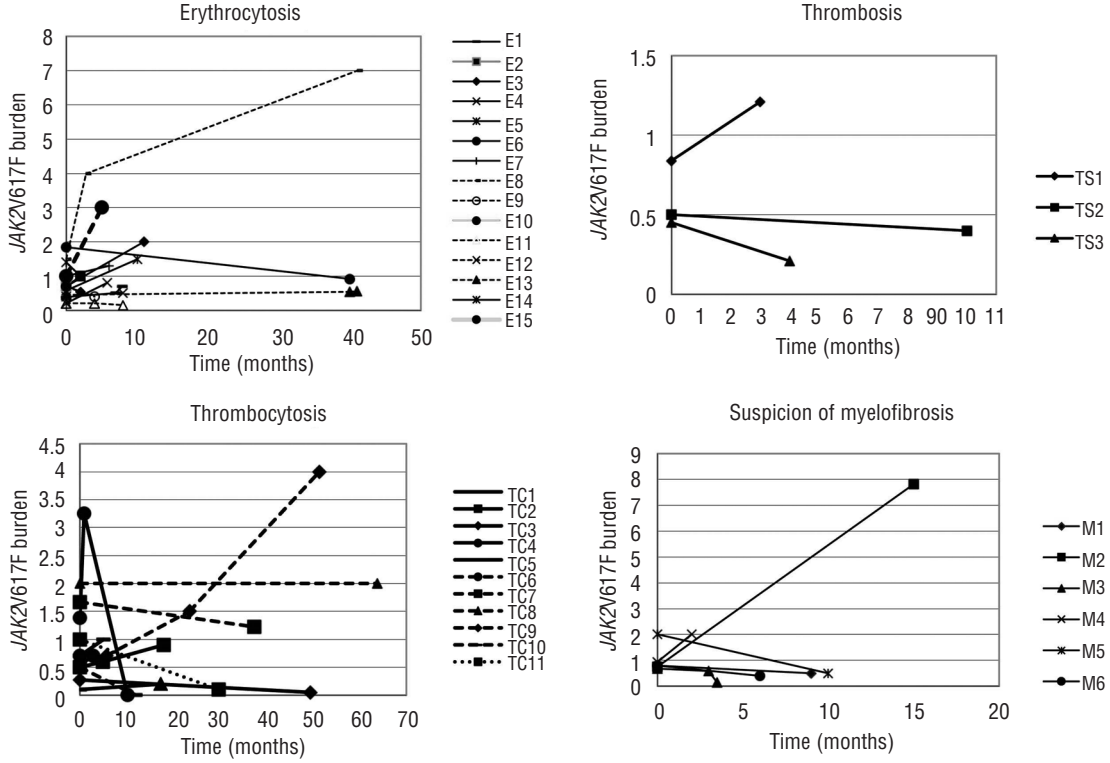

Figure 2. Evolution over time of the *JAK2*V617F allele burden of patients presenting with erythrocytosis, thromobosis, thrombocytosis or suspicion of myelofibrosis. Data generated by each laboratory are shown.

healthy elderly people.[12,13] Similarly, patients investigated for thrombosis did not have any evidence of hematologic malignancy.

In contrast, a diagnosis of hematologic malignancy was either confirmed or very likely for patients with thrombocytosis or/and suspicion of myelofibrosis, possibly because the vast majority of patients explored for thrombocytosis or suspicion of myelofibrosis do suffer from a hematologic malignancy, irrespective of mutational status. However, in our study, bone marrow histology was not in favor of MPN for all the patients presenting with thrombocytosis and a low *JAK2*V617F allele burden. This may be due to the fact that the patients were studied at early stages, had mild forms of disease and only minor histological features, not sufficient to establish a neoplastic myeloproliferation.

For all patients, the size of the clones was mostly stable over time (Figure 2) except for 2 patients with a hematologic malignancy: one presenting with erythrocytosis (patient E4) who was diagnosed with PV and whose allele burden reached 7% *JAK2*V617F, and one patient with myelofibrosis (patient M2) whose allele burden increased 10-fold. Of note, patient E15 tripled his *JAK2*V617F burden although he was finally diagnosed with false erythrocytosis. These observations confirm that a *JAK2*V617F -mutated clone can be present and can increase in individuals with no evidence of *MPN*.

In conclusion, finding a low allele burden of *JAK2*V617F should prompt the search for additional mutations: in the *JAK2* gene in case of erythrocytosis, and in the *CALR* and *MPL* genes in other cases with a suspicion of MPN. In the absence of additional mutations in *JAK2*, *MPL* or *CALR*, the significance of the *JAK2*V617F -mutated clone should not be considered to be sufficient evidence to establish malignant myeloproliferation. Long-term prospective studies should evaluate whether patients with minor *JAK2* (and possibly *MPL* or *CALR*) mutated clones and no overt MPN eventually develop full blown MPN and/or present complications, especially thrombotic events.

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