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ORIGINAL ARTICLE

A highly specific q-RT-PCR assay to address the relevance of the *JAK2WT* and *JAK2V617F* expression levels and control genes in *Ph*-negative myeloproliferative neoplasms

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Abstract In *Ph*− myeloproliferative neoplasms, the quantification of the *JAK2V617F* transcripts may provide some advantages over the DNA allele burden determination. We developed a q-RT-PCR to assess the *JAK2WT* and *JAK2V617F* mRNA expression in 105 cases (23 donors, 13 secondary polycythemia, 22 polycythemia vera (PV), 38 essential thrombocythemia (ET), and 9 primary myelofibrosis (PMF)). Compared with the standard allele-specific oligonucleotide (ASO)-PCR technique, our assay showed a 100 % concordance rate detecting the *JAK2V617F* mutation in 22/22 PV (100 %), 29/38 (76.3 %) ET, and 5/9 (55.5 %) PMF cases, respectively. The sensitivity of the assay was 0.01 %. Comparing DNA and RNA samples, we found that the *JAK2V617F* mutational ratios were significantly higher at the RNA level both in PV ($p=0.005$) and ET ($p=0.001$) samples. In PV patients, *JAK2WT* expression levels positively correlated with the platelets (PLTs) ($p=0.003$) whereas a trend to negative correlation was observed with the Hb levels ($p=0.051$). *JAK2V617F*-positive cases showed the lowest *JAK2WT* and *ABL1* mRNA expression levels. In all the samples, the expression pattern of *beta-glucoronidase* (*GUSB*) was more homogeneous than that of *ABL1* or β2 microglobulin (*B2M*). Using *GUSB* as normalizator gene, a significant increase of the *JAK2V617F* mRNA levels was seen in two ET patients at time of progression to PV. In conclusion, the proposed q-RT-PCR is a sensitive and accurate method to quantify the *JAK2* mutational status that can also show clinical correlations suggesting the impact of the residual amount of the *JAK2WT* allele on the *Ph*− MPN disease phenotype. Our observations also preclude the use of *ABL1* as a housekeeping gene for these neoplasms.

Keywords *Ph*− myeloproliferative neoplasms · *JAK2WT* level · *JAK2V617F* level · Housekeeping gene · q-RT-PCR

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Introduction

Philadelphia chromosome-negative myeloproliferative neoplasms (*Ph*− MPNs) are clonal myeloid disorders characterized

by an increased production of terminally differentiated cells. The mechanisms of MPN initiation and progression have been extensively studied. A number of genetic and epigenetic abnormalities associated with *Ph*− MPNs have been reported [1–5]. The somatic mutation of *JAK2V617F* is the most frequent genetic alteration in these diseases and is presently considered as a major diagnostic criterion for *Ph*− MPNs. The frequency of this mutation is greater than 95 % in patients with polycythemia vera (PV) and around 50 % in patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF).

JAK2V617F-positive *Ph*− MPNs show a biological continuum with clinical presentation, which is in part influenced by the *JAK2V617F* mutational load [6]. A relationship between *JAK2V617F* mutational burden and disease phenotype has been reported [7].

Although the clinical relevance of the correct quantification of the *JAK2* allele burden is still not clearly stated, the *JAK2* tyrosine kinase activity is now a therapeutic target for innovative and more specific treatment of these diseases [8]. The still debated pathogenesis of MPNs forces to seek methods which are increasingly specific and sensitive for the quantification of the *JAK2* allele burden at diagnosis and during treatment. To date, the detection and quantification of the *JAK2WT* and *JAK2V617F* alleles are usually assessed using genomic DNA. However, the quantification of *JAK2V617F* mRNA transcripts by a real-time quantitative RT-PCR (q-RT-PCR) may provide some advantages over the DNA allele burden [9]. In PV mononuclear cell samples, Zhao et al. [10] demonstrated that the ratio *JAK2V617F/JAK2WT* is higher in cDNA than in genomic DNA. Using an ARMS assay on cDNA originated from granulocyte mRNAs, Vannucchi et al. [11] identified more *JAK2*-mutated transcripts in MPN patients (9 %), as compared to conventional allele-specific PCR. Moreover, the mRNA (but not DNA) can be extracted from platelets present in buffy coats, and this may increase the *JAK2V617F* assay sensitivity in ET patient samples. Finally, in transgenic mice models, the *JAK2V617F* transcript levels were found to be strictly correlated with the *Ph*− MPN phenotypes [12]. By contrast, q-RT-PCR requires a parallel amplification of a housekeeping gene (HKG) as control gene (CG) to correct variations in RNA quality and quantity and to calculate the sensitivity of each measurement. Therefore, to better investigate the pathogenesis complexity of the *Ph*− MPNs and to provide a useful assay to monitor minimal residual disease (MRD), we set up an absolute q-RT-PCR method for the quantification of *JAK2WT* and *JAK2V617F* mRNA. The data herein reported show that this method is highly specific and sensitive. Moreover, the observation of a significant variability within the sample groups of the expression levels of *ABL1*, one of the most commonly used HKGs, leads us to test other genes as more appropriate CGs in this clinical setting.

Materials and methods

Patients and samples

Peripheral blood buffy coat specimens were collected from 105 individuals. Twenty-two of them were diagnosed as having PV, 38 ET, and 9 PMF according to the 2008 WHO diagnostic criteria [13]. Thirteen patients with secondary polycythemia (SP) and 23 healthy blood donors were included as controls. For three patients with initial diagnosis of ET who progressed to PV, paired samples of both disease phases were available. All specimens were collected after patients had signed an informed consent. The study was approved by our IRB at AUSL Latina (no. 6315/A001/2012).

DNA, RNA extraction, and cDNA synthesis

Cell pellets were either processed for DNA purification using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions, or resuspended in guanidine isothiocyanate for RNA extraction [14, 15]. Equal amounts of RNA (1 μg) were reverse transcribed into cDNAs with random hexamers and MuLV reverse transcriptase (Applied Biosystems, Monza, Italy).

JAK2V617F mutation analysis and allele burden assay

JAK2V617F mutation was detected in DNA samples using the allele-specific PCR method previously described by Baxter et al. [16] The quantification of *JAK2V617F* allele burden on genomic DNA was performed using the *JAK2V617F* MutaQuant® Allele kit (Qiagen, Milano, Italy), according to the manufacturer's instructions.

Allele-specific q-RT-PCR

To obtain reference curves, standard plasmids were manufactured to contain *JAK2WT* or *JAK2V617F* cDNA sequences. cDNAs were obtained from RNA extracted from human K562 cells (homozygous for *JAK2WT* allele) and HEL cells (homozygous for *JAK2V617F* allele). We designed specific primers (forward: TTCTGGATAAAGCACACAGAAA; reverse: CCAAATTTTACAAACTCCTGAACC), in order to amplify 150 bp including the V617F codon (NM_004972.3:c.1745-1895), using the Primer 3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). One-step cloning strategy was performed by using the TOPO TA Cloning® kit (Invitrogen, Monza, Italy). Each plasmid vector was checked by sequencing and includes five 10-fold serial dilutions, ranging from 10^5 to 10 targets per well.

JAK2WT and *JAK2V617F* mRNAs were measured as described by Merker et al. [17]. Primers and probe

concentrations were optimized at concentrations of 300 and 200 nM, respectively, in a final volume of 25 μl. q-RT-PCR was performed in the ABI 7900 (Applied Biosystems) with an initial 10-min incubation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples and no-template controls were run in triplicates.

For each sample, the *ABL1*, *B2M*, and *GUSB* copy numbers were determined by using standard endogenous plasmid controls (FusionQuant® Standards Ipsogen, Qiagen), according to the manufacturer's instructions and in accordance with the Europe Against Cancer (EAC) network [18].

JAK2 mutational burden evaluation

The mutational burden ratio of *JAK2* was calculated using the DNA and RNA copy numbers according to the following formula: *JAK2V617F*/(*JAK2V617F*+*JAK2WT*).

Statistical analysis

Statistical analyses and graphs were generated using SPSS-10 software. Further, targeted pairwise comparisons to evaluate the significance between individual diseases were performed by the two-sided Student's *t* test. Results were expressed as means ± SEM. The level of significance from *t* test was $p < 0.05$.

Results

Using the standard qualitative allele-specific oligonucleotide (ASO)-PCR method [17], the *JAK2V617F* was detected in 22/22 PV (100 %), in 29/38 (76.3 %) ET, and in 5/9 (55.5 %) PMF cases, respectively (Table 1). Our q-RT-PCR method confirmed the presence/absence of the *JAK2V617F* mutation in all the cases, resulting in a 100 % concordance rate with the standard assay. In addition, we did not observe nonspecific amplification in either *JAK2WT* or *JAK2V617F* plasmids or in positive or negative controls and *JAK2V617F*-positive and negative samples (Fig. S1 A, B). cDNA carrying a predetermined *JAK2V617F* copy number was diluted with cDNA from a healthy control to establish the sensitivity of our q-RT-PCR method. Serial dilution experiments demonstrated a detection limit of 0.01 % (Fig. S2 A, B).

The main demographic, clinical, and laboratory characteristics of donors and patients, grouped according to initial diagnosis, together with their *JAK2WT*, *JAK2V617F*, *ABL1*, *B2M*, *and GUSB* median absolute copy numbers are reported in Table 1. Several lines of evidence demonstrate that the transition between PV, ET, and PMF is frequent in *JAK2V617F*-positive *Ph*− MPN patients, suggesting for these disease entities a biologic continuum [6]. Therefore, the analysis of the expression levels of the *JAK2WT*, *ABL1*, *B2M*, and *GUSB* genes was done, grouping *Ph*− MPN cases according to the presence/absence of the *JAK2V617F* mutation.

The median of absolute *JAK2WT* mRNA copy numbers detected in the donor, SP, and *JAK2V617F*-positive and *JAK2V617F*-negative *Ph*− MPN groups is depicted in Fig. 1a. *JAK2V617F*-positive patients presented a significantly lower *JAK2WT* mRNA expression compared with donors and SP and *JAK2V617F*-negative patients ($p=0.002$; $p<0.001$; $p<0.001$, respectively). Significantly higher *JAK2V617F* mRNA expression levels were detectable in PMF patients when compared with ET cases; differences between PV and ET samples were not statistically significant (Fig. 1b).

In a group of 12 PV, 17 ET, and 3 PMF, we matched the *JAK2V617F* mutational ratios, in patient-derived DNA and RNA samples. As shown in Fig. 2, with respect to DNA, ratios were significantly higher when assessed at the RNA level both in PV ($p=0.005$) and ET ($p=0.001$) samples and not statistically significant in PMFs (three cases). The comparisons of the DNA or RNA *JAK2V617F* mutational burden ratios showed significant differences whether they were calculated at the DNA or RNA level. At the DNA level, ET patients showed significantly lower *JAK2V617F* allele burden ratios as compared to both PV and PMF (22 % vs. 45 % and vs. 47 %, respectively [ET vs. PV $p=0.01$; ET vs. PMF $p=0.02$]) whereas, at the RNA level, significant differences were recorded only between ET and PMF patient groups (56 vs. 80 %; $p=0.03$) (Fig. 2).

We did not observe significant associations correlating the *JAK2WT* or *JAK2V617F* expression levels and Hb, PLTs, WBC, splenomegaly, or occurrence of thrombosis in the entire patient group. Instead, considering the sole PV group, we observed a positive statistically significant correlation between the *JAK2WT* expression levels and PLT count ($R^2=0.39$; $p=0.003$) and a trend to negative correlation between the *JAK2WT* expression levels and the Hb levels ($R^2=0.10$; $p=0.051$) (Fig. 3a, b).

We also found that the *ABL1* gene was not uniformly expressed among healthy donors and patient groups, being expressed at higher levels in donors and SP and *JAK2V617F*-negative patients compared to *JAK2V617F*-positive cases (Fig. 4a). A significant positive correlation between the copy numbers of *ABL1* and *JAK2WT* was measured in *JAK2V617F*-positive patients (Fig. 4b). These observations raise several doubts about the appropriateness of using *ABL1* as CG in *Ph*− MPN patients. Therefore, to identify CGs more suitable than *ABL1*, using the q-RT-PCR protocols optimized by the EAC program, we evaluated the *B2M* and *GUSB* expression levels in a group of 68 samples (donors=11, SP=5, PV=16, ET=31, and PMF=5) with remaining available RNA. Both *B2M* and *GUSB* showed similar expression levels within all the groups examined. However, *GUSB* showed the most homogeneous medians and the narrowest distribution of

Table 1 The diagnostic main clinical–hematologic characteristics of the 105 individuals included in the present study grouped according to diagnosis

Characteristics	Donors ($n=23$)	SP ($n=13$)	PV ($n=22$)	ET ($n=38$)		PMF ($n=9$)	
			JAK2WT/V617F ($n=0$) ($n=22$; 100 %)	JAK2WT/V617F ($n=9$; 19.5 %) ($n=29$; 80.5 %)		JAK2WT/V617F ($n=4$) ($n=5$)	
Age, median (years) (range)	45 (26–61)	59 (27–90)	65 (52–88)	63 (35–80)	62 (38–81)	75 (70–80)	68 (47–83)
Gender, male/female	16/7	13/0	17/5	4/5	20/29	2/4	3/5
Hb (g/dl), median (range)	15 (11.5–16.9)	17 (16–18.2)	18 (17.6–20.2)	12.5 (11.6–14)	15 (12–19)	9.6 (8.9–10)	15.4 (13.8–17.3)
WBC (1×10^9/l), median (range)	6.6 (4.7–9.3)	7.1 (4.8–11.4)	10.6 (4.4–15)	8.5 (6–10)	9.9 (7.1–18)	8 (1.3–16.8)	20.6 (20–10.8)
PLTs (1×10^9/l), median (range)	232 (192–349)	200.5 (91–274)	370 (75–712)	792 (631–1,420)	718 (256–1,318)	364 (129–489)	555 (197–946)
Neutrophils (1×10^9/l), median (range)	4.4 (1.4–7)	4.6 (1.4–7.5)	7.9 (2.7–13)	4.6 (2.8–7.6)	6 (1.4–13.5)	5.1 (1–9.4)	15.9 (8.4–19.5)
JAK2WT copy numbers (1×10^3), median (range)	7.7 (1.2–38.5)	8.7 (2.5–30.9)	6.9 (0.02–22.6)	15.6 (6.2–34.5)	6.0 (0.9–16.7)	11.2 (3.8–18.5)	3.0 (0.3–4.5)
JAK2V617F copy numbers (1×10^3), median (range)	–	–	26.9 (0.2–203.6)	–	6.9 (0.7–52.8)	–	29.4 (1.4–78.7)
ABL1 copy number (1×10^3), median (range)	4.6 (1.6–11.9)	5.0 (2.4–20.6)	2.9 (0.9–12.7)	7.4 (1.8–17.7)	3.8 (0.3–8.7)	8.6 (4.6–14.9)	3.3 (1.7–4.6)
B2M copy number (1×10^6), median (range)[a]	8.0 (2.8–10.1)	4.8 (2.5–13.0)	8.8 (2.4–17.7)	6.1 (2.4–7.9)	8.1 (2.4–17.3)	6.0	7.0 (2.3–13.4)
GUSB copy number (1×10^3), median (range)[a]	37.6 (7.4–53.2)	34.5 (3.2–58.7)	37.8 (0.2–56.7)	39.8 (8.6–71.6)	33.2 (5.8–59.1)	23.4	41.0 (19.3–93.5)

[a] *B2M* and *GUSB* copy numbers were detected in donor ($n=11$), SP ($n=5$), PV ($n=16$), ET (WT $n=7$; V617F $n=24$), and PMF (WT $n=1$; V617F $n=4$) samples

values also in the *JAJ2V617F*-positive cases (Fig. 5a, b) where the expression levels of *ABL1* were significantly affected. In 11 control samples, we assessed the Ct values of *ABL1*, *B2M*, *GUSB*, and *JAK2* genes. In all cases, the variations in Ct values turned out to be comparable, and all fell within three Ct values. However, the Ct values of *B2M* were lower than those of *ABL1*, *GUSB*, and *JAK2*, thus indicating the higher level of expression of *B2M* in these samples (Fig. S3).

In three ET patients, who progressed to PV during this study, we could serially assess at ET diagnosis and PV progression the DNA and RNA *JAK2V617F* mutational ratios and the *JAK2V617F* expression levels normalized with *GUS*. The clinical–biologic parameters of these three patients are reported in Table 2. As illustrated in Fig. 6, we observed a significant increase of the *JAK2V617F* expression levels at PV progression in two of the three cases (case 1 and 3, Fig. 6c) and a trend toward statistical significance in the remaining

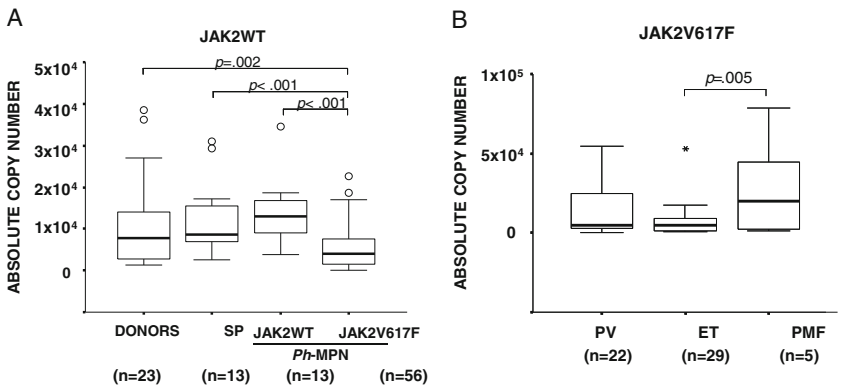

Fig. 1 *JAK2WT* and *JAK2V617F* mRNA expression levels detected by q-RT-PCR method. **a** *JAK2WT* absolute copy numbers, in donors, SP cases, and *Ph*− MPN patients. **b** *JAK2V617F* absolute copy numbers in *JAK2V617F*-positive *Ph*− MPN patients. *Circles* indicate the outlier values. *Asterisks* indicate the extreme values

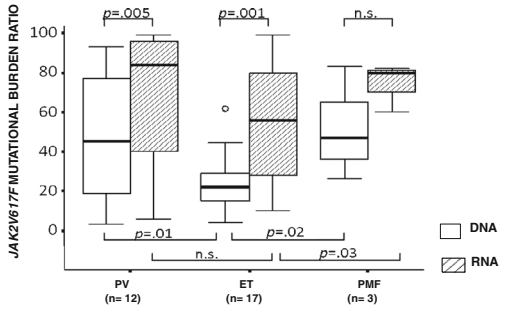

Fig. 2 *JAK2V617F* mutational burden ratio, calculated at the DNA and RNA levels. The *circle* indicates the outlier values

case. By contrast, the JAK2 mutational ratios at DNA and RNA were increased only in case 1 (Fig. 6a, b and Table 2).

Discussion

Our study shows that, in the diagnostic approach to *Ph*− MPN patients, a q-RT-PCR assay to detect *JAK2V617F* expression levels offers comparable specificity than ASO-PCR and allele burden quantitative assays. Moreover, this method is probably more sensitive than these "standard" methods, as supported by the fact that the *JAK2V617F* mutational ratios were steadily superior at the RNA than at the DNA level in all the groups studied. These latter findings, showing an impact of the type of nucleic acid on the determination of the *JAK2V617F* burden, are in apparent contrast to those reported by Vannucchi et al. [11] who did not see differences with respect to the use of RNA or DNA. A likely explanation of these discrepancies may rely on the fact that we used buffy-coat preparations, which include platelets instead of the isolated leukocytes. These findings might also suggest that, using RNA, it is possible to avoid the cost-effective and time-consuming methods for leukocyte isolation.

The higher sensitivity to detect the *JAK2V617F* mutation at the RNA level was firstly demonstrated by the above mentioned study of Vannucchi et al. [11], who reported an increased percentage (9 %) of *JAK2*-mutated ET patients by using RNA instead of DNA. Thus, the mRNA template should be recommended for the diagnosis of ET patients, usually presenting a lower *JAK2V617F* allele burden, or in the monitoring of *Ph*− MPN treatment response.

The present q-RT-PCR method was derived from that recently proposed by Merker et al. [17] for the quantification of the *JAK2WT* and *JAK2V617F* transcript levels in *Ph*− MPNs. However, after testing more than 100 samples of patients with and without *Ph*− MPNs, these authors reported a low level of nonspecific amplifications in samples containing a high copy number of standard plasmids and in blood specimens from patients without *Ph*− MPNs. To eliminate these undesired amplifications, Merker et al. [17] established a mutant to wild type cutoff of <0.0005. We optimized Merker's amplification reaction by reducing the amount of final concentrations of primers from 800 to 300 nM and of probes to 400 to 200 nM, respectively. We did not observe nonspecific amplifications in *JAK2WT* or *JAK2V617F* reaction by using plasmid standards, K562 and HEL cell lines, or *JAK2V617F*-positive and negative samples, tested as positive or negative controls (Fig. S1 A, B). The lack of nonspecific amplifications justifies the use of this test for diagnostic purposes.

Although the analysis of DNA is in principle technically simpler than that of RNA, discrepant results are often reported even after DNA-based techniques, so that Lippert et al. [19] have emphasized the need of using positive and negative quality controls, and calibration to a reference standard to improve reproducibility. More recently, the European Leukemia Net/MPN&MPNr-EuroNet group, to avoid that the variations in the performance of the plethora of qPCR assays routinely used to detect *JAK2V617F* could potentially impact on their clinical utility, selected the most sensitive and performing of nine DNA-based quantitative PCR assays as the optimal quantitative-polymerase chain reaction method for routine diagnosis and tracking of minimal residual disease in *JAK2V617F*-associated myeloproliferative neoplasms [20].

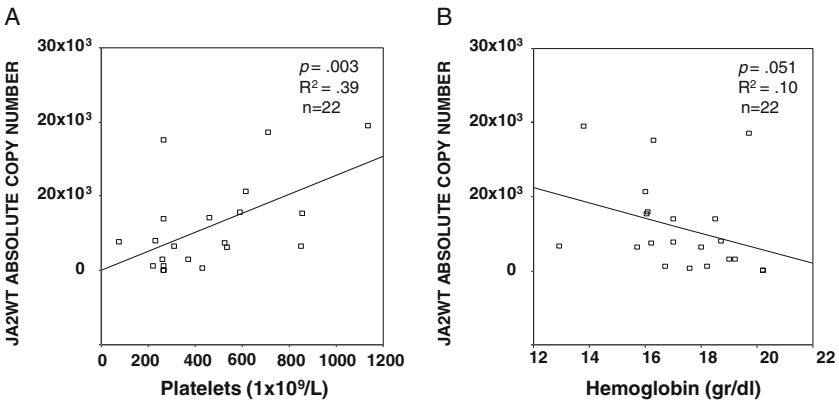

Fig. 3 Correlations between the *JAK2WT* expression levels and PLT count (**a**) and Hb levels (**b**) in the PV patient group

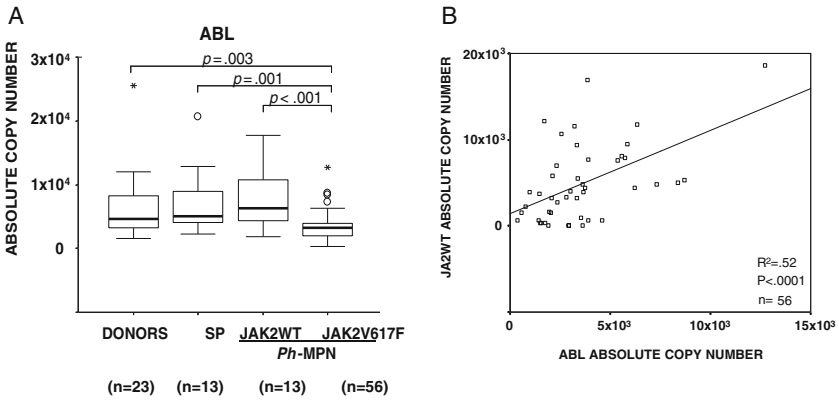

Fig. 4 **a** *ABL-1* absolute copy numbers in donors, SP cases, and *Ph*− MPN patients grouped according to the presence/absence of the *JAK2V617F* mutation. *Circles* indicate the outlier values. The *asterisks* indicate the extreme values. **b** Correlation between the *ABL-1* and *JAK2WT* expression levels in the *JAK2V617F*-positive *Ph*− MPN patient group

Unlike reports measuring DNA levels, we did not detect higher *JAK2V617F* mRNA expression levels in samples from ET patients with respect to PV. One possible explanation of this discrepancy may be that the higher sensitivity of the present q-RT-PCR method, by increasing the *JAK2V617F* copy numbers detected in ET samples, has canceled this difference.

To evaluate the putative prognostic value of the *JAK2WT* and *JAK2V617F* expression levels detected by the present assay, we search for significant correlations with the patient's clinical–biologic parameters. The relatively small number of cases did not allow us to draw conclusive remarks on this issue. However, it is interesting to note that in PV patients, we observed a significant correlation between the *JAK2WT* expression levels and PLT count and a trend to a negative correlation with the Hb values. These latter findings may confirm the assumptions that even the residual amount of the normal *JAK2* allele may impact on the different *Ph*-negative MPN entities [21].

Aiming to set up a sensitive q-RT-PCR method allowing the quantification of gene expression, and having to compensate for potential variability of this procedure, we firstly used *ABL1* as HKG. However, we found that the *ABL1* gene was not uniformly expressed among donor and *JAK2V617F*-negative and positive patient groups, being significantly lower in the group characterized by the *JAK2V617F* mutation. This finding clearly contradicts the most important requisite of a HKG that should be expressed at a similar level in all the tested samples. Therefore, although *ABL1* is one of the most commonly used HKG in hematologic neoplasms [22], at least in *Ph*− MPNs, it should not be chosen for gene expression analysis. To identify CGs more suitable than *ABL1*, we evaluated two other widely used HKGs as *B2M* and *GUSB*. Both these genes showed similar expression levels within all the groups examined, but *GUSB* showed the most homogeneous medians and the narrowest distribution of values also in the JAK2V617F-positive cases (Fig. 4). Moreover, we confirmed that in control samples, *JAK2*, *ABL1*, and *B2M* and *GUSB* have comparable Ct variations, falling in all cases within three values and that *B2M*, compared to the other three genes, is clearly overexpressed (Fig.S3). The fact that these latter data exactly reproduced those achieved by the EAC program in a much larger number of cases than ours confirmed their reliability. Based on these observations, we would suggest *GUSB*, but not *ABL1*, as the most appropriate GC for this clinical setting.

The observed positive correlation between the expression levels of *JAK2WT* and *ABL1* in the *JAK2V617F*-positive patient group further suggests the issue that *JAK2V617F*-positive MPN cases may represent a distinct biologic entity.

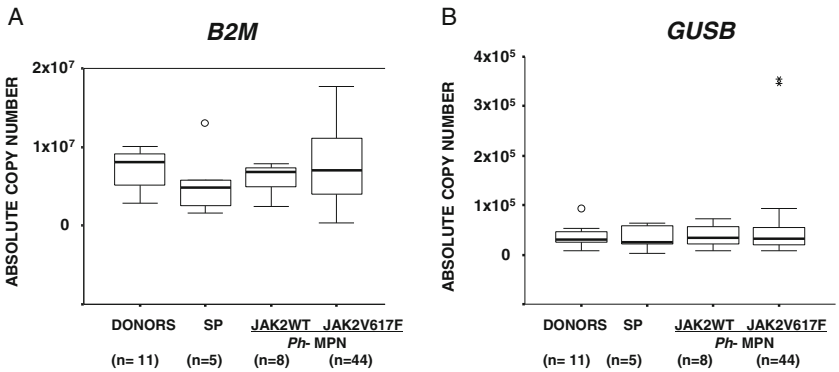

Fig. 5 *B2M* (**a**) and *GUSB* (**b**) absolute copy numbers in donors, SP cases, and *Ph*− MPN patients grouped according to the presence/absence of the *JAK2V617F* mutation. The *circle* indicates the outlier values. The *asterisks* indicate the extreme values

Table 2 The hematologic characteristics of the three ET patients who progressed to PV referred at the time of ET diagnosis and at PV progression (pPV)

Characteristics	Case 1		Case 2		Case 3	
	ET	pPV	ET	pPV	ET	pPV
WBC (1×10^9/l), median (range)	9.9	26.2	7.1	12	9.2	10.69
PLTs (1×10^9/l), median (range)	696	1,003	1,184	1,500	519	516
Neutrophils (1×10^9/l), median (range)	7.5	21	5.6	7.6	5.4	6.73
JAK2WT copy number (1×10^3), mean ± SEM	5.8±0.7	1±0.01	5.4±0.25	3.7±0.2	3.2±0.08	7.1±1
JAK2V617F copy number (1×10^3), mean ± SEM	1.3±0.3	5.7±0.5	2.6±0.1	3.6±0.6	4.1±0.5	10.2±0.3
DNA mutational burden ratio	22	69	29.4	28	24	21
mRNA mutational burden ratio	18	85	32	49	56	59
B2M copy number (1×10^3), mean ± SEM	1,751±27	2,256±500	1,974±75	1,953±71	1,376±142	1,749±163
GUSB copy number (1×10^3), mean ± SEM	15.5±0.6	10.4±1.1	15.6±0.8	16.1±0.3	7.0±0.06	6.9±0.2

In addition, this finding may support the model recently proposed by Irino et al. [23]. These authors, focusing on genes involved in the JAK-STAT signaling pathway, identified two upregulated genes in MPN patients: *SOCS3*, a known target of the JAK-STAT axis and a potentially novel target, and *SPI1*, encoding *PU.1*. The latter gene is a regulator of proliferation and differentiation of hematopoietic cells [24]. The pathogenic effect of *JAK2* mutation appears mediated, at least in part, through the upregulation of *PU.1*. In addition, they showed that *PU.1* is regulated by both *JAK2* and *ABL1*, and suggested that *SOCS3* and *PU.1* are common downstream targets of both *JAK2* and *ABL1*. Together, these observations and our results link the constitutive activation of *JAK2* to the downregulation of the expression of *ABL1*.

The potential usefulness of *GUSB* as CG might also be supported by the additional findings observed in the three ET patients who progressed to PV. The serial monitoring of the DNA and RNA *JAK2V617F* mutational ratios and the *JAK2V617F* expression levels normalized with *GUSB* allowed to observe a significant increase of the *JAK2V617F* expression levels at PV progression in two of the three cases (case 1 and 3, Fig. 6c) and a trend toward statistical significance in the remaining case. By contrast, the *JAK2* mutational ratios at DNA and RNA were increased only in case 1 (Fig. 6a, b). These data support in humans the findings recently demonstrated in transgenic mice showing that the levels of *JAK2V617F* expression influence the *Ph*− MPN phenotype: higher levels favor erythrocytosis whereas lower levels favor thrombocytosis [12]. Similarly, Barosi et al. observed a progression toward JAK2V617F homozygosity in serial DNA samples collected from 64 patients with PMF [25].

In conclusion, the q-RT-PCR assay hereby reported is a sensitive and accurate method to quantify the *JAK2* mutational status that can also show clinical correlations suggesting the impact of the residual amount of the *JAK2WT* allele on the *Ph*− MPN disease phenotype. In addition, for the first time, we provided evidences that *ABL1* is not a useful CG to obtain reliable *JAK2V617F* quantifications in *Ph*− MPN patients whereas *GUSB* turned out be more appropriate for this purpose. These findings might become clinically relevant in light of the availability of several new and effective targeted therapies which require sensitive and precise assessment of the patient's response to treatments.

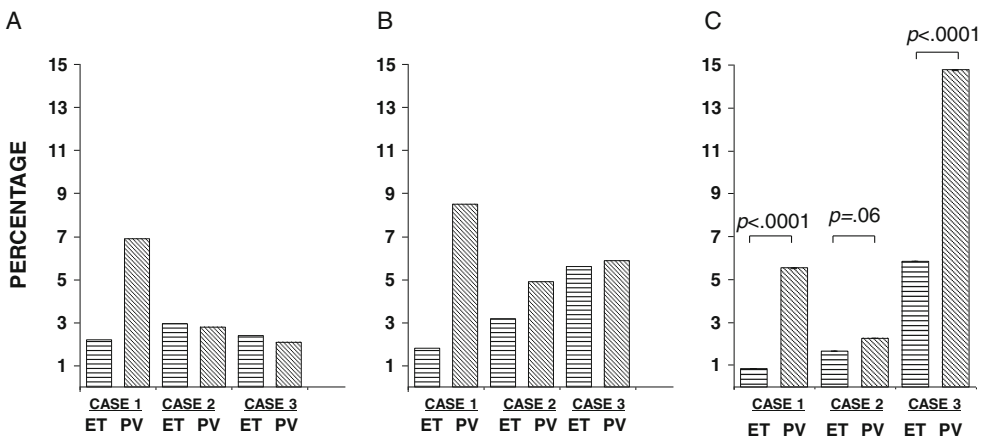

Fig. 6 The *JAK2V617F* mutational burden ratio in the three ET patients who progressed to PV evaluated by the following three different methods: **a** the absolute allele-specific PCR, **b** the present absolute allele-specific q-RT-PCR, and **c** using *GUSB* to normalize the values achieved by the present q-RT-PCR assay

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Conflict of interest The authors report no potential conflicts of interest.

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