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Myeloproliferative neoplasms are clonal hematopoietic stem cell disorders characterized by aberrant proliferation and an increased tendency toward leukemic transformation. The genes *JAK2*, *MPL,* and *CALR* are frequently altered in these syndromes, and their mutations are often a strong argument for diagnosis. We analyzed the mutational profiles of these three genes in a cohort of 164 suspected myeloproliferative neoplasms. *JAK2 V617F* mutation was detected by real-time PCR, whereas high-resolution melting analysis followed by Sanger sequencing were used for searching for mutations in *JAK2* exon 12, *CALR,* and *MPL*. *JAK2 V617F* mutation was associated with *CALR* ($n = 4$) and *MPL* ($n = 4$) mutations in 8 of 103 essential thrombocytosis patients. These cases were harboring a *JAK2 V617F* allelic burden of <4% and a significantly higher platelet count compared with *JAK2 V617F* ($P < 0.001$) and *CALR* ($P = 0.001$) single-mutation patients. The findings from this study support the possibility of coexisting mutations of the *JAK2*, *CALR,* and *MPL* genes in myeloproliferative neoplasms and suggest that *CALR* and *MPL* should be analyzed not only in *JAK2*-negative patients but also in low V617F mutation patients. Follow-up of these double-mutation cases will be important for determining whether this group of patients presents particular evolution or complications.

The Philadelphia-negative myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by excessive proliferation of one or several myeloid lineages. According to the 2008 World Health Organization classification of hematologic tumors,[1] revised in 2016,[2] these disorders include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF). The biological confirmations of these pathologies, which have long been diagnoses of exclusion, largely rely on the determination of the mutational status of three genes: *JAK2* (Janus kinase 2), *MPL* (myeloproliferative leukemia virus), and *CALR* (calreticulin).

JAK2 V617F mutation was the first clonal anomaly detected in Philadelphia-negative MPNs.[3–6] This valine-to-phenylalanine substitution at amino acid position 617 in exon 14 results in constitutive activation of the JAK2 tyrosine kinase.[3–7] *JAK2 V617F* mutation is found in >95% of patients with PV and in 56% to 65% of those with ET and PMF. Other *JAK2* mutations located in exon 12 can be found in the rare cases of PV lacking the V617F mutation.[8,9]

Several qualitative or quantitative methods of detecting *JAK2 V617F* mutation have been developed, such as restriction fragment length polymorphism, allele-specific amplification combined with real-time PCR, high-resolution melting (HRM), Sanger sequencing, and pyrosequencing.[3,10,11] Allele-specific amplification combined with real-time PCR is the assay widely most used for detecting this mutation with high sensitivity.[12] The results are generally reported as percentage of *JAK2 V617F* allele copy number over total (mutant and wild-type) allele copy number.

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CALR mutations were recently identified as driver mutations in Philadelphia-negative MPN and constitute the second most frequent molecular anomalies in ET and PMF patients, with a prevalence of approximately 20% to 35% of cases.[13–15] With the exception of isolated reports,[16] these mutations appear to be relatively specific to ET and PMF.[17–19]

The third frequent mutation in MPN involves the *MPL* gene, most often at codon position 515. Several substitutions have been described at this position.[20–22] *MPL* mutations are present in 2% to 10% of patients with ET and PMF,[23] and only rarely in patients with PV.[22]

JAK2, *CALR,* and *MPL* mutations are generally described as mutually exclusive, and the following diagnostic algorithm is classically used for stratifying the molecular analysis to be done when such a syndrome is suspected. Briefly, the first mutations to search for should be *JAK2 V617F* mutations. If the results are negative, then a second line of analysis should search for mutations in *JAK2* exon 12 if a PV diagnosis is suspected or in *CALR* exon 9 and *MPL* exon 10 if the suspected disease is TE or PMF.

However, some rare cases of double mutations have been reported, with *JAK2 V617F* being associated with either a *CALR*[13,24] or *MPL* mutation.[25] Interestingly, Nussenzveig et al[25] recently reported an increased frequency of *JAK2* exon 12 and *MPL* mutations in patients presenting low *JAK2 V617F* allelic burden. To investigate the possible association of mutations of these three genes in MPN, we analyzed mutational profiles in a cohort composed of 164 suspected MPN cases harboring at least one mutation of these genes.

Materials and Methods

Population

A total of 169 MPN-suspected patients harboring at least one mutation in *JAK2*, *CALR,* or *MPL*, detected between January 2001 and December 2015 at the Biochemistry and Molecular Biology Unit of North Hospital (Marseilles, France), and for which genomic DNA was available, were included in this study. Concerning cases of *JAK2 V617F* mutation with an allelic burden of <4% and *CALR* and *MPL* mutation, all of the positive cases over this period were included in this study. This 4% cutoff was arbitrarily chosen as some widely used tests for *JAK2 V617F* present a lower limit of detection of between 2% and 4%. In *JAK2 V617F* mutation cases harboring an allelic burden of >4%, we included a sampling of patients whose allelic burdens ranged from 4% to 89.3% analyzed over the same period. All patients provided written informed consent.

DNA Extraction

Genomic DNA was isolated from 200 μL of total blood with the EZ1 DNA Blood 200 μL kit (Qiagen, Les Ulis, France) using the EZ1 Advanced XL instrument (Qiagen) according to the manufacturer's recommendations. DNA was quantified with a, NanoDrop 8000 spectrophotometer (Thermo Scientific, Illkirch, France), and diluted to 5 ng/μL in DNase- and RNase-free water.

JAK2 V617F Quantification

Real-time fluorescent quantitative PCR using allele-specific primer and TaqMan-MGB probe for dual-inhibiting amplification of wild-type alleles were performed for the detection of as low as 0.05% of mutant *JAK2 V617F* cells. All of the samples were tested in duplicate using the Mx 3000P real-time PCR system (Agilent Technologies, Les Ulis, France). The TaqMan assay amplification was performed with 25 ng of genomic DNA in 1× TaqMan Universal PCR Master Mix (Life Technologies, Villebon-Sur-Yvette, France). The primers and probe sequences and concentrations used for the quantification of *JAK2 V617F* alleles were as follows: 600 nmol/L of forward primer (5′-AGCTTTCTCACAAGCATTTGGTT-3′), 300 nmol/L of reverse primer (5′-GTTTTACTTACTCTCGTCTCCACAAAA-3′), and 200 nmol/L of probe (5′-6FAM-AATTATGGAGTATGTTTCTGMGBFNQ-3′). The amplification of the albumin gene (*Alb*) as internal control was performed with 300 nmol/L of each primer (Fw: 5′-ATGCTGCACAGAATCCTTGGT-3′; Rv: 5′-TCATCGACTTCCAGAGCTGAAA-3′) and 200 nmol/L of probe (5′-6VIC-AACAGGCGACCATGC-MGBBFNQ-3′). The thermal cycling conditions were 95°C for 10 minutes, and 45 cycles at 95°C for 30 seconds and 60°C for 1 minute. The *JAK2 V617F* and *Alb* copy numbers were obtained from a plasmid calibrator standard curve. The mutational burden ratio of *JAK2* was calculated using the DNA copy numbers according to the formula *JAK2 V617F/Alb*.

HRM Analysis

The HRM assays were performed using the LightCycler 480 instrument (Roche Diagnostics, Meylan, France). All samples were tested in duplicate, and one wild-type DNA control and two positive controls were included in each experiment and for each gene. A quantity of 25 ng of DNA was amplified in a final volume of 20 μL containing 1× LightCycler 480 High Resolution Melting PCR Master Mix (Roche Diagnostics), 300 nmol/L of each primer, and 3 mmol/L $MgCl_2$. Primer sequences were as follows: *CALR*-Fw, 5′-CCCTGAGGTGTGTGCTCTG-3′ and *CALR*-Rv, 5′-GAGGCAGGCCTCTCTACAGC-3′ for *CALR* exon 9; and, as previously published,[26,27] *MPL*-Fw, 5′-GCCGAAGTCTGACCCTTTTT-3′ and *MPL*-Rv, 5′-ACAGAGCGAACCAAGAATGCCTGTTTACA-3′ for *MPL* exon 10; JAKex12Fw, 5′-ACCAACCTCACCAACATTACAGAG-3′ and JAKex12Rv, 5′-AAAAGGACAAAAAAGACAGTAATGAGTATC-3′ for *JAK2* exon 12. LC480 cycling parameters were as follows: initial denaturation at 95°C for 10 minutes; 45 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 25 seconds.

Table 1 Demographic and Hematologic Characteristics of Included Patients

Characteristic	PV	ET	PMF	Unclassified MPN
Number (%) of patients	28 (17)	103 (64)	11 (7)	22 (13)
Numbers of men/women	7/21	44/59	4/8	12/10
Age, median (range), years				
All patients	60 (24–89)	63 (25–89)	69 (41–83)	74 (24–89)
Women	65 (24–89)	61 (25–89)	64 (41–78)	65 (34–85)
Men	56 (26–80)	65 (26–89)	73 (54–83)	59 (18–82)
Hemoglobin median (range), g/L				
All patients	172 (145–224)	133 (78–164)	107 (83–133)	139 (94–191)
Women	159 (145–224)	130 (78–154)	129 (90–133)	133 (94–167)
Men	177 (169–189)	137 (81–164)	106 (83–130)	164 (94–191)
White blood cell count, median (range), $\times 10^9$/L	9.1 (4.3–17.3)	8.36 (3.49–21.0)	6.40 (1.5–26.9)	8.5 (3.0–13.0)
Platelet count, median (range), $\times 10^9$/L	264 (159–866)	710 (467–2300)	187 (19–864)	325 (14–1239)
Number of mutated cases				
JAK2 V617F	25	56	8	20
JAK2 exon 12	3	0	0	1
CALR exon 9	0	48	2	0
MPL exon 10	0	7	1	1
JAK2 V617F allele burden, mean (range), %	11.82 (0.08–56.67)	13.83 (0.06–89.29)	18.14 (0.11–52.56)	0.53 (0.05–34.08)

ET, essential thrombocythemia; MPN, myeloproliferative neoplasms; PMF, primary myelofibrosis; PV, polycythemia vera.

The final melting program was denaturation at 95°C for 1 minute, renaturation at 40°C for 1 minute, and melting from 65°C to 95°C, with a ramp of 0.02°C/second and 25 fluorescence acquisitions per degree centigrade. Results were analyzed by Gene Scanning software version 1.5 (Roche Diagnostics). The lower limit of detection of this in-house method was 5%.

Sequencing

Sanger sequencing was performed on the same amplicons as used for HRM analysis. A quantity of 1 µL of PCR product was purified with ExoSap-IT (Affymetrix USB; Fisher Scientific, Illkirch, France). Unidirectional sequencing reaction was set up with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies). The BigDye XTerminator Purification Kit (Life Technologies) was used for purifying the reaction products. Sequencing analyses were performed on the ABI Prism 3130 Genetic Analyzer (Life Technologies).

Statistical Analysis

For nonparametric continuous variables, the Mann-Whitney *U*-test was used. Significance was set at a *P* value of 0.05. Sex ratio distribution was analyzed using a Pearson χ^2 test. All statistical analyses were performed using SPSS software version 20.0 (SPSS, Bologna, Italy).

Cloning

The amplicon used for HRM analysis was cloned using the TOPO-TA kit as described by the manufacturer (Life Technologies). Recombinant plasmids were isolated, and bidirectional sequencing was performed to validate the sequences on an ABI Prism 3130 Genetic Analyzer (Life Technologies) as described in *Sequencing*.

Results

To search for coexisting mutations in the *JAK2*, *CALR*, and *MPL* genes, we constituted a cohort of 164 patients with suspected MPN analyzed between January 1, 2013, and December 31, 2015, who had a mutation in at least one of these genes. For the purposes of this study, we completed the mutation profiles of these patients by analyzing exons 12, 9, and 10 of the *JAK2*, *CALR*, and *MPL* genes, respectively, if not done previously, as those mutations are usually researched only in wild-type *JAK2* cases. Among

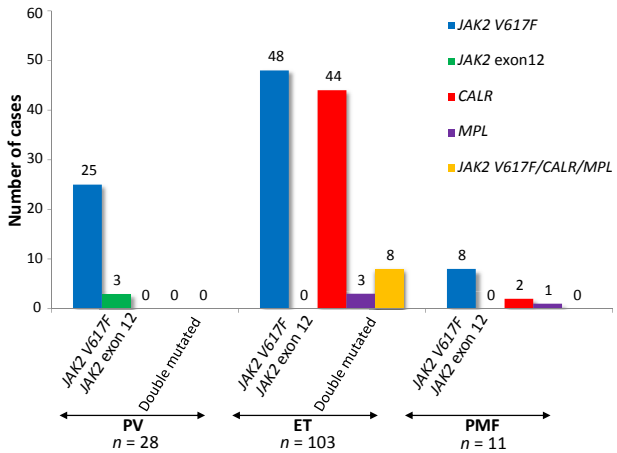

Figure 1 Mutational profiles of myeloproliferative neoplasm–diagnosed patients. ET, essential thrombocythemia; PMF, primary myelofibrosis; PV, polycythemia vera.

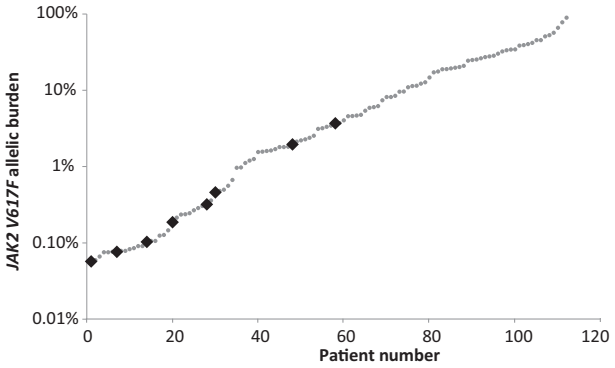

Figure 2 *JAK2 V617F* allelic burden distribution. Each circle represents one *JAK2 V617F* mutation case. Diamonds represent patients in whom *JAK2 V617F* mutation is associated with *CALR* or *MPL* mutation.

those patients, 142 were diagnosed as having MPN according to the World Health Organization criteria (except for ET cases in which the bone marrow biopsy was classified as restricted−uncertain diagnosis only). The 22 remaining cases did not display enough criteria for a specific MPN subtype and were classified as MPN−unclassifiable. Most of these cases were referred to our laboratory for thrombosis events exploration. ET, PV, and PMF were diagnosed in 103 (72.5%), 28 (19.7%), and 11 (7.7%) patients, respectively. Clinical and hematologic data are described in Table 1.

V617F mutation was tested using an in-house quantitative PCR assay with a lower limit of detection of 0.05%. A total of 109 patients harbored a V617F allelic burden comprising between 0.05% and 89.3% (Figures 1 and 2). The mean values of these ratios were 13.8%, 11.8%, and 18.1% in ET, PV, and PMF, respectively. Those differences were not significant when comparing PV with ET ($P = 0.096$), PV with PMF ($P = 0.176$), or ET with PMF ($P = 0.49$).

HRM was used for analyzing exons 12, 9, and 10 of the *JAK2*, *CALR* (Figure 3A), and *MPL* (Figure 3B) genes, respectively. Characterization of mutations was achieved by Sanger sequencing in cases showing HRM curve variation. *JAK2* exon 12 mutations were already described and found in three cases of PV (c.1611-1616del6, c.1627-1632del, and c.1615_1616AA>TT) and in one post-PV myelofibrosis (c.1613_1616 ACAA>T). *CALR* exon 9 mutation was detected in 51 cases. Type 1 and type 2 represented, respectively, 37.3% ($n = 20$) and 41.2% ($n = 22$) of *CALR* mutations. The remaining eight *CALR* mutations were indel events previously described in the literature.[14] Among those *CALR* mutation patients, ET and PMF were diagnosed in 49 and 2 cases, respectively. Finally, 11 patients harbored an *MPL* sequence variation. Two of these variations corresponded to one previously described intronic substitution (c.1565+5 C>T) reported as a germline probably benign mutation.[28] Among the nine other sequence variations, seven were classic tryptophan 515 substitutions and two were a less-characterized V501A substitutions. All of these *MPL* mutation patients, except one harboring V501A substitution, were diagnosed as having ET.

In our cohort, *JAK2 V617F* mutation was associated with *MPL* exon 10 in four cases and to *CALR* exon 9 mutations in four cases. Clinical and hematologic characteristics of these double-mutation cases are depicted in Table 2. All coexisting mutations were detected in ET cases, with a median platelet count of 1120×10^9 platelets/L. *JAK2* exon 12 mutations were not associated with other mutations, and *CALR* and *MPL* mutations were not associated with each other. The four *MPL* mutations were three W515L and one W515R substitution in patients with *JAK2 V617F* allelic burdens of 0.08%, 0.10%, 3.69%, and 0.32%, respectively. *CALR* mutations were c.1154_1155insTTGTC (type 2) in two cases, c.1095_1140del (type 3) in one case, and c.1125_1147del in one case, and these four mutations were associated with V617F allelic burdens of 0.06%, 0.19%, 1.96, and 0.46%, respectively. Hence, all of the coexisting mutations were detected in patients with a V617F allelic burden of <4% (Figure 2). Conversely, in all except one case of double mutation, mutations in *MPL* and *CALR* genes were unambiguously detected by HRM analysis and Sanger sequencing, suggesting an allelic burden of ≥10%. In one of these double-mutation patients, *MPL W515L* substitution was detected by HRM analysis, but sequencing confirmation required TA Cloning (Thermo Fisher Scientific) (Figure 3, C−E).

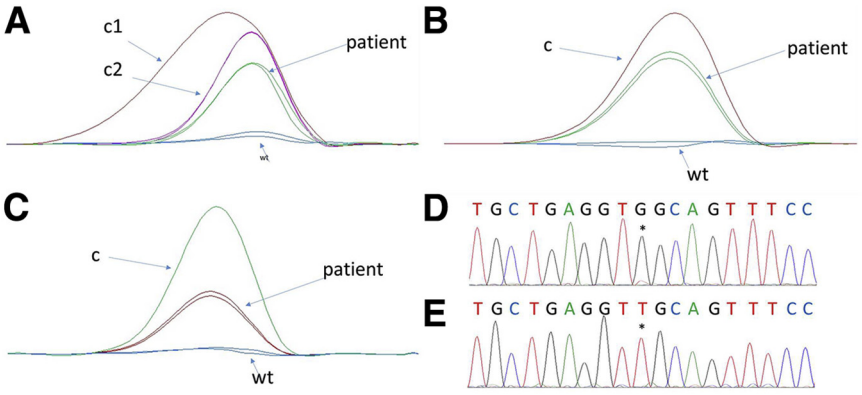

Figure 3 High-resolution melting (HRM) and sequencing results. **A:** *CALR* HRM analysis. c1 and c2 curves correspond to type 1 and type 2 *CALR* mutation controls, respectively; wt curve, wild-type control. **B:** *MPL* HRM analysis. c curve corresponds to W515L mutation control; wt curve, wild-type control. **C:** W515L mutation case detected by HRM analysis but in which sequencing needed TA Cloning (Thermo Fisher Scientific), as presented in **D** (before cloning) and **E** (after cloning). The **asterisks** indicate the locations of the point mutations.

Table 2 Characteristics of *JAK2 V617F*—Associated Mutations

	CALR mutations in *JAK2* mutation cases				*MPL* mutations in *JAK2* mutation cases			
Characteristic	c.1154_1155insTTGTC	c.1154_1155insTTGTC	c.1095_1140del	c.1125_1147del	W515L	W515L	W515L	W515R
Age, years	70	27	89	73	53	75	54	69
Sex	M	F	F	F	F	F	F	F
Hemoglobin level, g/L	117	134	111	149	110	98	98	135
WBC count, $\times 10^9$/L	-	6.61	9.75	7.98	13.88	11.57	11.41	12.59
Platelet count, $\times 10^9$/L	947	912	1162	1241	1078	2300	1863	1050
JAK2 V617F allele burden	0.06%	0.19%	1.96%	0.46%	0.08	0.10	3.69	0.32

WBC, white blood cell.

Biological and clinical characteristics of single- and double-mutation patients were compared (Table 3). As double-mutation patients were all diagnosed with ET, only ET cases were taken into account for this statistical analysis. The three cases of *MPL* single-mutation patients were not considered for statistical comparison. As expected, *CALR* single-mutation patients displayed a significantly higher platelet count compared with that in *JAK2 V617F* mutation patients ($P = 0.009$). On the other hand, hemoglobin levels were significantly higher in *JAK2* mutation patients, but this difference was restricted to females only ($P = 0.006$). Platelet count was significantly higher in double-mutation patients compared with that in single-mutation *JAK2 V617F* ($P < 0.001$) or *CALR* ($P = 0.001$) cases, reaching 2300×10^9 platelets/L in one *JAK2 V617F*/*MPL* mutation case. No statistical difference was seen when comparing age or white blood cell counts between these different groups.

Discussion

JAK2 V617F mutation is the most frequent mutation in MPN, occurring in >95% patients with PV and in approximately 50% of patients with ET or PMF. Due to this high mutation frequency in MPN, the *JAK2 V617F* assay is the first step in the molecular diagnosis of these syndromes, and it is widely agreed that when V6147F mutation is detected, no further molecular analysis is required for characterizing the disease. V617F allelic burden level is highly variable,[29–32] with ET usually harboring the lowest values compared with PV and PMF.[33,34] Several tests are available for analyzing *JAK2 V617F* mutation with very variable sensitivity, with some techniques allowing for the detection of as low as 0.005% of *JAK2 V617F* allelic burden.[35] However, the clinical significance of low V617F allelic burden is not clear.[36]

Table 3 Clinical and Laboratory Characteristics of 103 ET Patients, Stratified by Mutation Profile, and Statistical Comparison between These Groups of Patients

	Single- or double-mutation ET patients						
	Group 1	Group 2	Group3	Group 4	*P*		
	JAK2 V617F ($n = 48$)	*CALR* ($n = 45$)	*MPL* ($n = 3$)	Double-mutation cases ($n = 8$)	1 vs. 2	1 vs. 4	2 vs. 4
Age median (range), years	63 (25–89)	62 (26–89)	57 (46–64)	70 (27–89)			
Sex ratio F/M	1	1.4	2	7			
White blood cell count, median (range), $\times 10^9$/L	8.86 (3.39–21.00)	7.84 (4.30 23.00)	6.69 (6.54–12.06)	11.41 (6.61–13.88)			
Hemoglobin, median (range), g/L							
All patients	139 (81–162)	130 (78–161)	136 (124–164)	114 (98–149)			
Women	140.5 (97–154) ($n = 24$)	127 (78–152) ($n = 26$)	130 (124–136) ($n = 2$)	111 (98–149) ($n = 7$)	0.006		
Men	128 (81–162) ($n = 24$)	142 (110–161) ($n = 18$)	164 ($n = 1$)	117 ($n = 1$)		N/A	N/A
Platelet count, median (range), $\times 10^9$/L	631 (467–1981)	783 (471–1638)	712 (519–1033)	1120 (912–2300)	0.009	<0.001	0.001

ET, essential thrombocythemia; N/A, not applicable.

In this study, we included 169 patients harboring at least one mutation in the *JAK2*, *CALR,* or *MPL* gene, and completed the mutational profiles of these patients by analyzing these three genes in each case. Among those cases, 22 were identified as MPN−unclassifiable due to insufficient classic MPN criteria. In most of these cases, *JAK2 V617F* allelic burden was highly variable, ranging from 0.05% to 89.3%. A total of 142 patients were diagnosed as having MPN (28 PV, 103 ET, and 11 PMF). PV is usually reported to present the highest V617F allelic burden, whereas PMF exhibits lower values of this ratio.[35,37,38] However, the reported values of this allelic burden are highly variable among the different publications. We did not find any significant difference in *JAK2 V617F* allelic burden between PV, ET, and PMF cases in our cohort. This finding could have been due to a selection bias, as we included all of the cases of low V617F allelic burden detected over the 3-year observation period but only a sampling of higher mutation rates, ranging from 4% to 89.3%. This observation will have to be verified in larger-scale cohorts.

The overall frequency of the coexisting mutations in this cohort was estimated to be 4.9% (8/164). *JAK2* exon 12 mutations were not associated with other mutations of the genes analyzed in this study, including 28 PV cases, which may be insufficient for detecting those rare events. *CALR* ($n = 4$) or *MPL* ($n = 4$) mutation was associated with *JAK2 V617F* mutation in eight ET cases. Those cases were restricted to ET-diagnosed patients harboring a low *JAK2 V617F* allelic burden, <4%. MPN diagnosis may be uncertain in cases in which low *JAK2 V617F* allelic burden is associated with atypical clinical features. The findings from this study indicate that the analysis of *CALR* and *MPL* mutational statuses might be useful in these situations.

These double-mutation cases seem to display particular characteristics differentiating them from single-mutation MPNs. Platelet counts were significantly higher in patients in whom coexisting mutations were detected, which may lead to several complications, such as thrombotic or hemorrhagic events. Our study cohort was too small to investigate this question, which will be of particular interest. Among the eight double-mutation cases, seven were women. Added to the low *JAK2 V617F* allelic burden, these characteristics could point out a particular group of patients, and this concept will need to be evaluated in larger cohorts.

In the double-mutation cases, *CALR* or *MPL* mutations were detected at a significantly higher rate than were V617F mutations. This finding might be explained by any of at least three hypotheses:

i) The 5% lower limit of detection of the HRM method may have made it impossible to detect low *CALR* or *MPL* allelic burden. Nonetheless, in double-mutation cases, Sanger sequencing profiles suggested *CALR* and *MPL* mutation burdens of >10%.
ii) The two different mutations may be present in two different pathologic clones.
iii) *CALR* or *MPL* mutation may be a secondary event conferring a selective advantage to those pathologic clones. A way to answer this question might be to apply higher-sensitivity methods, such as single-cell analysis or culture isolation. However, in one *JAK2 V617F*/*MPL* mutation patient, the signal corresponding to the mutant *MPL* gene analyzed by Sanger sequencing was at the lower limit of detection of this technique, meaning that cells bearing this mutation were less abundant than in the other double-mutation cases. Thus, at least *MPL* mutation can be present at low allelic burden as well as *JAK2 V617F*. The clinical significance of these low abundant clones is not well understood[36] and has to be clarified for physicians to know how to manage these patients. If the detection of these low-mutation patients proves to be interesting, techniques with higher sensitivity such as quantitative PCR or next-generation sequencing will have to be used for searching for such rare events.

In conclusion, the findings from this study support the possibility of coexisting mutations of the *JAK2*, *CALR,* and/or *MPL* genes in MPN and suggest that *CALR* and *MPL* should be analyzed not only in *JAK2*-negative patients but also in low V617F mutation patients, at least in those in whom clinical diagnosis is difficult. Further investigation in larger-scale cohorts will be needed for confirming that these coexisting mutations are restricted to low *JAK2 V617F* mutation cases and for analyzing the prognostic value of these events.

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