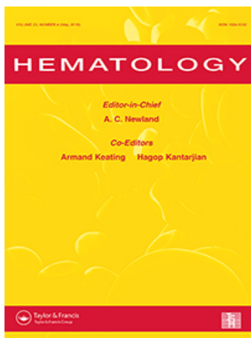

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CALR, *JAK2* and *MPL* mutation status in Argentinean patients with *BCR-ABL1*-negative myeloproliferative neoplasms

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ABSTRACT

Objectives: To establish the frequency of *JAK2*, *MPL* and *CALR* mutations in Argentinean patients with *BCR-ABL1*-negative myeloproliferative neoplasms (MPN) and to compare their clinical and haematological features.

Methods: Mutations of *JAK2*V617F, *JAK2* exon 12, *MPL* W515L/K and *CALR* were analysed in 439 Argentinean patients with *BCR-ABL1*-negative MPN, including 176 polycythemia vera (PV), 214 essential thrombocythemia (ET) and 49 primary myelofibrosis (PMF).

Results: In 94.9% of PV, 85.5% ET and 85.2% PMF, we found mutations in *JAK2*, *MPL* or *CALR*. 74.9% carried *JAK2*V617F, 12.3% *CALR* mutations, 2.1% *MPL* mutations and 10.7% were triple negative. In ET, nine types of *CALR* mutations were identified, four of which were novel. PMF patients were limited to types 1 and 2, type 2 being more frequent.

Discussion: In ET, patients with *CALR* mutation were younger and had higher platelet counts than those with *JAK2*V617F and triple negative. In addition, *JAK2*V617F patients had high leucocyte and haemoglobin values compared with *CALR*-mutated and triple-negative patients. In PMF, patients with mutant *CALR* were associated with higher platelet counts.

Conclusion: Our study underscores the importance of *JAK2*, *MPL* and *CALR* genotyping for accurate diagnosis of patients with *BCR-ABL1*-negative MPN.

KEYWORDS
Myeloproliferative neoplasms; polycythemia vera; essential thrombocythemia; primary myelofibrosis; calreticulin; *JAK2*V617F;*MPL*

Introduction

The most frequent *BCR-ABL1*-negative myeloproliferative neoplasms (MPN) include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), according to the 2008 World Health Organization (WHO) classification criteria [1]. The understanding of the genetic basis of these disorders began in 2005, when the *JAK2*V617F mutation was described as the first recurrent molecular abnormality in *BCR-ABL1*-negative MPN [2–6]. This mutation is present in about 95% of patients with PV and in about 50–60% of those with ET or PMF. Mutations in *JAK2* exon 12 and *MPL* exon 10 were subsequently reported in subsets of patients. *JAK2* exon 12 mutations have been detected in most of the remaining cases with PV [7–10], while *MPL* exon 10 mutations (mainly involving codon W515) occur in 5–10% of patients with *JAK2*V617F-negative ET or PMF [11–14]. In 2013, somatic mutations in CALR gene, encoding calreticulin, were found in most patients with ET or PMF with *JAK2* and *MPL* wild-type, in a mutually exclusive pattern with *JAK2* and *MPL* mutations. Mutant *CALR* is the result of frameshift mutations caused by exon 9 deletions or insertions, type-1, 52-bp deletion (p.L367fs*46), and type-2, 5-bp TTGTC insertion (p.K385fs*47) variants constitute more than 80% of these mutations [15–17].

The aim of the present study is to establish the frequency of major mutations in the *JAK2*, *MPL* and *CALR* genes in Argentinean patients with *BCR-ABL1*-negative MPN. The clinical and haematological features of ET and PMF patients were also compared according to the driver mutations displayed.

Materials and methods

This study was approved by the ethical committee at Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after patients had provided written informed consent.

From January 2008 to December 2016, bone marrow or peripheral blood samples from 439 *BCR-ABL1*-negative MPN patients were referred to our centre. According to the 2008 WHO criteria [2], 176 out of 439 subjects had PV, 214 had ET and 49 PMF. For each patient, a sample of genomic DNA was isolated and relevant laboratory information at the time of diagnosis was obtained by reviewing their case history.

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*JAK2*V617F mutation was assessed in all patients by a tetra-primer PCR assay, as previously described. [18] Patients with PV and negative *JAK2*V617F were sequenced to screen for *JAK2* exon 12 mutation [7]. Patients with non-mutated *JAK2* PMF or ET were further evaluated for *MPL* exon 10 mutations. An allele-specific PCR assay was designed to detect *MPL* W515L and W515 K. In three separate reactions, a 346-bp fragment was amplified with the following primers: W515 forward specific primer CTGCTGCTGCT GAGGTG, L515 forward specific primer CTGCTGCTGCTG AGGTT, K515 forward specific primer CTGCTGCTGCTG AGGAA, and the common reverse primer AGGAGGT GGGACTGACG. Each reaction was performed from 50 to 200 ng of DNA in a final volume of 25 µl containing 0.4 µmol/L of each primer (Operon), dNTPs 0.2 mM (Promega), Cl_2Mg 2.5 mM (Promega), Taq 1× buffer (Promega) and 1 U of DNA Taq polymerase (Promega). The PCR used the following cycle protocol: an initial denaturation at 95°C for 5 minutes was followed by 30 cycles at 95°C for 30 seconds, 60°C for 60 seconds and 72°C for 45 seconds, with a final elongation cycle at 72°C for 3 minutes. This assay was further validated by Sanger sequencing to determine the reliability of the method in a routine clinical setting (data not shown). In order to detect insertions or deletions in the exon 9 of *CALR* gene in patients with non-mutated *JAK2* and *MPL*, the complete sequence of this exon was amplified by PCR and sequenced as previously described [16].

All the laboratory parameters included in the statistical analyses were gathered at diagnosis. Differences in the distribution of continuous variables between categories were analysed by either Mann–Whitney or Kruskal–Wallis tests. Patient groups with nominal variables were compared by the Chi-square test or Fisher's exact test, when appropriate. All *P*-values were considered statistically significant when smaller than 0.05 (two-tailed). Statistical analyses were performed using the GraphPad Prism software, version 5.0 (GraphPad software, Inc., San Diego, CA, USA). Numerical variables have been summarized by their median and range, and categorical variables by count and relative frequency (%) of each category.

Results

As reported in Table 1, out of 439 patients studied, 329 (74.9%) carried *JAK2*V617F, 54 (12.3%) a *CALR* exon 9 frameshift mutation, 9 (2.1%) a *MPL* mutation, and 47 (10.7%) had non-mutated *JAK2*, *MPL* and *CALR* (i.e. triple-negative patients). We did not find any *JAK2* exon 12 mutations. The positivity of the *JAK2*V617F mutation was 94.9% in PV, 61.2% in ET and 62% in PMF. *MPL* exon 10 mutations were found in 6% of ET and 2% of PMF. *CALR* exon 9 mutations were detected in ET and PMF, with mutational frequencies of 21.5 and 18%, respectively.

Out of the nine subjects with *MPL* mutations, seven carried W515L (five ET and two PMF subjects) and two carried W515 K mutation (one ET and one PMF subjects). Among subjects with *CALR* mutations, type 1 (c.1092_1143del) and type 2 (c.1154_1155insTTGTC) occurred in 20 cases (37%) and 24 cases (44.4%), respectively. In the remaining 10 cases (18.6%), seven variant mutations were found: c1095_1140del (4 cases), c.1118_1136del (one case), c.1125_1146del (one case), as well as four cases of previously unrecognized mutations (c.1094_1130del, c.1112_1154del, c1127_1134delinsTCTTTGCTTA and c.1149_1154delins TCCTCCTTGTC). In ET, type 1 was found in 19 cases (41.3%), type 2 in 17 (37%) and the other seven types were present in 10 cases (21.7%), while in PMF, type 1 was found in 1 case (12.5%) and type 2 in 7 (87.5%) (Table 1).

Table 2 reports the main clinical and haematological features at diagnosis of the ET patients studied according to their genotype. The *MPL* group was excluded from the statistical analysis due to its small size. *CALR* frameshift mutations were associated with

Table 1. Frequency of driver mutations in *BCR-ABL1*-negative MPNs.

	PV (*n* = 176) (%)	ET (*n* = 214) (%)	PMF (*n* = 49) (%)	MPN (*n* = 439) (%)
JAK2 V617F	167 (94.9)	131 (61.2)	31 (62)	329 (74.9)
CALR mutations	–	46 (21.5)	8 (16.3)	54 (12.3)
Type 1 mutation	–	19 (41.3)	1 (12.5)	20 (37)
Type 2 mutation	–	17 (37)	7 (87.5)	24 (44.4)
MPL mutation	–	6 (2.8)	3 (6.1)	9 (2.1)
Triple negative	9 (5.1)	31 (14.5)	7 (14.3)	47 (10.7)

Table 2. Main clinical and hematological features of 208 patients with ET stratified by mutational status.

	*JAK2*V617F[1]	*CALR*[2]	Triple negative[3]	$P^{1vs.2}$ value	$P^{1vs.3}$ value	$P^{2vs.3}$ value
N	131	46	31			
Sex (male/female)	51/80 (40%/60%)	20/26 (44%/56%)	10/21 (32%/68%)	0.604	0.542	0.35
Age onset (years)	64 (54–75)	59 (47–69)	48 (32–66)	0.168	**0.012**	0.212
Leucocytes (×10^9/L)	11.3 (9.2–15.2)	8.3 (7.3–10.3)	9.6 (7.7–12.1)	**<0.001**	**<0.001**	0.237
Haemoglobin (g/dL)	14 (12.2–15.4)	12.6 (11.6–14.0)	12.8 (11.3–14.3)	**<0.001**	**0.009**	0.79
Platelets (×10^9/L)	885 (654–1123)	1028 (889–1509)	860 (720–1200)	**<0.001**	0.49	**0.009**

Statistically significant values ($P < 0.05$) are shown in bold.

Table 3. Main clinical and hematological features of 46 patients with PMF stratified by mutational status.

	JAK617F[1]	*CALR*[2]	Triple negative[3]	$P^{1vs.2}$ value	$P^{1vs.3}$ value	$P^{2vs.3}$ value
N	31	8	7			
Sex (male/female)	13/18 (42%/58%)	03/05 (38%–62%)	07/00 (100%–0%)	–	–	–
Age onset (years)	64 (57–76)	65 (51–72)	58 (57–76)	0.93	0.699	0.855
Leucocytes ($\times10^9$/L)	17 (8.2–29)	11.2 (3.9–19)	12.9 (2.9–32)	0.114	0.374	0.698
Haemoglobin (g/dL)	11 (9.5–13)	9.4 (7.5–13.2)	9.3 (8.4–11.3)	0.342	0.247	0.948
Platelets ($\times10^9$/L)	276 (145–505)	529.5 (199–951)	80 (54–112)	0.142	**0.009**	**<0.001**

Statistically significant values ($P < 0.05$) are shown in bold.

lower white blood cell counts, lower haemoglobin levels, higher platelet counts and lower age compared with *JAK2* mutations. Triple-negative patients showed lower age at presentation, lower white blood cell counts, lower haemoglobin levels compared with patients with *JAK2* mutations and had lower platelet counts than those with *CALR* mutations. In PMF, patients with mutant *CALR* were associated with higher platelet counts (Table 3).

Discussion

In this study, we found *JAK2*, *MPL* or *CALR* mutations in 94.9% of the cases with PV, 85.5% in patients with ET and 85.2% with PMF. Hence, the combined genetic tests of these driver mutations are essential for accurate diagnoses of *BCR-ABL1*-negative MPN. *JAK2*V617F mutation was the most frequent in the 3 subtypes of MPN studied followed by *CALR* mutations in ET and PMF. Among those patients without *JAK2* or *MPL* mutations, the frequency of *CALR* frameshift mutations was 59.7% in ET and 53.3% in PMF, these findings being consistent with previous reports [15–16].

Nine different types of *CALR* exon 9 mutations were identified including deletions, insertions and complex indels. 81.4% of patients carried the most frequently described type 1 or 2 and the remaining 18.6% carried seven other types, four of which were novel. All novel mutations resulted in a common +1 bp-altered reading frame and predicted a novel C-terminal peptide sequence lacking the KDEL motif, as is the case for other mutations. Patients with PMF were limited to types 1 and 2, type 2 mutation (seven cases) being more frequent than the type 1 mutation (one case).

Although type 1 mutation was mainly associated with a myelofibrosis phenotype, [16,19–21] we found a significantly higher frequency in ET than in PMF (41.3 vs. 12.5%), as also observed in Chinese patients [22,23]. This may be attributed to the differences in the diagnostic criteria and ethnic origin.

When comparing the clinical and haematological characteristics of patients with ET, it was observed that patients with *CALR* exon 9 mutations were statistically younger than those with *JAK2*V617F. Triple-negative patients showed a tendency to be the youngest though there was no statistical significance between these patients and those with *CALR* mutations. In addition, *JAK2*V617F patients had high leucocyte and haemoglobin values compared to *CALR*-mutated and triple-negative patients. *CALR*-mutated patients had high platelet counts compared with *JAK2*V617F and triple-negative patients. Contrary to other studies, [24–26] we did not find a male predilection in *CALR*-mutated group. In the case of patients with PMF, it has been reported that *CALR* mutations are correlated with younger age, higher platelet count, lower leucocyte count and higher haemoglobin level [19,27]. We found that *CALR* patients had higher platelet count than those with *JAK2*V617F and triple-negative patients, although there was no statistical significance between *CALR*-mutated and *JAK2*V617F patients. In addition, *JAK2*V617F patients showed a tendency to high leucocyte and haemoglobin values compared to *CALR*-mutated and triple-negative patients. We also observed a male predilection and a tendency to be younger in triple-negative patients, but this should be confirmed by further studies with higher number of patients.

In conclusion, our findings indicate that the mutation profile of our *BCR-ABL1*-negative MPN patients is in line with previous reports. In addition, our data show similarities with these reports regarding clinical and haematological features of ET patients. However, we observed differences in the ratio of *CALR* mutation types and in clinical and haematological features in PMF. Probably, these discrepancies were due to the small size of *CALR*-mutated and triple-negative PMF subgroups. Finally, our study underscores the importance of *JAK2*, *MPL* and *CALR* genotyping for an accurate diagnosis of patients with *BCR-ABL1*-negative MPN.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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