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CALR mutations screening in wild type *JAK2*^{V617F} and *MPL*$^{W515K/L}$ Brazilian myeloproliferative neoplasm patients

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ABSTRACT

Some myeloproliferative neoplasm (MPN) patients harbor *JAK2*^{V617F} mutation, and *CALR* mutations were recently discovered in wild type (WT) *JAK2*^{V617F}. We evaluated the frequency and type of *CALR* mutations, and clinical and hematological characteristics in WT *JAK2*^{V617F} and *MPL*$^{W515K/L}$ MPN patients. Sixty-five patients were included: 21 with primary myelofibrosis (PMF), 21 with myelofibrosis post-essential thrombocythemia (MPET) and 23 with essential thrombocythemia (ET). Screening for *JAK2*^{V617F} and *MPL*$^{W515K/L}$ were performed using real-time PCR, while *CALR* mutations were analyzed by fragment analysis and Sanger sequencing. *JAK2*^{V617F} was the most frequent mutation (54.5%) and one patient (1.5%) harbored *MPL*^{W515L}. *CALR* mutations were present in 38.1% of PMF, 12.5% of ET and 33.3% of MPET patients. Five types of *CALR* mutations were detected, among which type 1 (32.1%) and type 2 (21.4%) were found to be the most common. A novel *CALR* mutation in a PMF patient was found. Patients carrying *CALR* mutations had higher platelet count and less presence of splenomegaly than *JAK2*^{V617F}, while triple negatives had higher C-reactive protein levels than *CALR* mutant carriers. Screening for *CALR* mutations and its correlation with clinical features could be useful for the characterization of MPN patients and result in its incorporation into a new prognostic score.

1. Introduction

Chronic myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders characterized by aberrant expansion of one or more myeloid lineages. The most common Philadelphia-negative MPNs are primary myelofibrosis (PMF), essential thrombocythemia (ET) and polycythemia vera (PV) [1].

The identification of a single base substitution in *JAK2*, the gene encoding the protein janus kinase 2, provided the first molecular biomarker of these MPN. *JAK2*^{V617F} mutation was found in 95% of patients with PV and in 60% to 65% of those with PMF and ET [2–5]. The remaining 5% of patients with PV carry a somatic mutation of *JAK2* exon 12 [6,7]. Subsequent studies showed that somatic mutations of *MPL* exon 10, mainly involving codon W515, are found in about 5% of PMF and ET patients [8–10]. Additional mutations were also identified in MPN patients but they affect only a small minority.

Recently, two studies identified mutations in *CALR* exon 9 gene in the 70 to 84% of wild type (WT) *JAK2*^{V617F} and *MPL*$^{W515K/L}$ PMF and ET patients providing strong genetic evidence that *CALR* mutations have an important role in the pathogenesis of these disorders [11,12]. These mutations are insertions or deletions leading to a frameshift responsible for modifying the C-terminal part of the calreticulin protein. The last four amino acids of calreticulin (KDEL), which contain the endoplasmic reticulum-retention signal, becomes positively charged and the reticulum targeting KDEL sequence is abrogated, which disturbs its cellular localization [11].

The frequency of *CALR* mutations is about 20% to 25% of all patients with PMF and ET [11,12]. To date, more than 50 different types of mutations in *CALR* have been detected, but type 1 variant (p.L367fs*46) resulting from 52 bp deletion and type 2 variant (p.K385fs*47) resulting from 5 bp TTGTC insertion are the most frequent types, overall being found in more than 80% of all patients with mutant *CALR* [13].

Since *CALR* mutations discovery, several studies have correlated these mutations with clinical data and patient outcome in MPNs [14–24]. Interestingly, the presence of *CALR* mutation in peripheral granulocytes of two PV patients negative for both *JAK2*^{V617F} and *JAK2*^{exon12} mutations [25] was described. The coexistence of *JAK2*^{V617F} and *CALR* mutations have been found in rare cases of refractory

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anemia with ringed sideroblasts associated with marked thrombocytosis [26], PMF [16,27], ET [28–30] and PV [30].

The identification and evaluation of disease-specific mutations could be valuable for diagnosis, prognosis and monitoring of treatment response. In the present study the frequency and types of *CALR* mutations were described, and hematological and clinical features of *JAK2*^{V617F} patients were compared with those carrying *CALR* mutations and triple negatives.

2. Patients and methods

2.1. Patients and study design

This study enrolled 65 MPN patients, consisting of 21 PMF and 23 ET diagnosed according to World Health Organization (WHO) criteria (2008) [31]. Other 21 myelofibrosis post-essential thrombocythemia (MPET) patients were diagnosed according to the International Working Group for MPN Research and Treatment [32]. Patients were selected from the Discipline of Hematology and Hemotherapy from the Universidade Federal de São Paulo and from the Hematology Division of the Universidade Católica de São Paulo, Brazil. Clinical and laboratory parameters were obtained at the time of the first diagnosis or when peripheral blood for genomic DNA samples was collected. Hematological analysis was conducted by Cell-Dyn 3700 (Abbott Diagnostics, CA, USA) and C-reactive protein levels were determined by ultrasensitive immunoturbidimetry. Genomic DNA samples were tested at the Fleury Group Center, in a laboratory certified according to the provisions of the College of American Pathologists (CAP), ISO-9001, ISO-14001 and the Brazilian Clinical Laboratory Accreditation Program (PALC). This study was conducted in accordance with the guidelines of the Institutional Ethics Committee.

2.2. Detection of JAK2, MPL and CALR mutations

Genomic DNA was isolated from peripheral blood leukocytes using a QIAamp Blood Mini Kit DNA extraction (PreAnalytix/Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Screening for *JAK2*^{V617F} (and allele burden) and *MPL*$^{W515K/L}$ mutations were performed using TaqMan-based real-time PCR method as previously described [33]. Only WT *JAK2*^{V617F} samples were analyzed for *MPL*$^{W515K/L}$ mutations. The ones without *MPL*$^{W515K/L}$ mutations were further analyzed for *CALR* mutations.

CALR exon 9 mutations were examined by fragment analysis using high resolution sizing of fluorescence-labeled PCR products. A PCR reaction was set up with a M13F (−21) sequence attached to the forward primer (TGTAAAACGACGGCCAGTCTGGCACCATCTTTGACAACTT) and a M13R (2) attached to the reverse primer (CAGGAAACAGCTATGACCGG CCTCTCTACAGCTCGTC), besides a M13F primer (TGTAAAACGACGGC CAGT) attached to a FAM fluorophore.

PCR reactions were prepared using 10 μL of PCR mix (2×) (Promega, Madison WI, USA), 0.15 μM of each primer, 5 μL of DNA template and water to a final reaction volume of 20 μL. The PCR cycling conditions were: an initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 96 °C for 30 s, annealing at 57 °C for 30 s, plus 20 cycles of annealing at 53 °C, extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. Fragment products were analyzed by capillary electrophoresis (Applied Biosystems 3500; Applied Biosystems, Foster City/CA, EUA) and evaluated by GeneMapper® ID-X Software Version 1.2 —) Applied Biosystems, Foster City/CA, EUA. All the samples were further analyzed by Sanger sequencing. A PCR reaction was set up with the same forward and reverse primers described above. The PCR final volume and concentrations were the same as for the previous reaction, and the PCR cycling excluded the annealing temperature to 53 °C in the last 20 cycles. Mutations were identified using CLC Genomics Workbench (Qiagen, Aarhus, Denmark), and the secondary structure of the protein was predicted using EMBOSS Transeq (EMBL-EBI, UK) (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and JPRED [33].

2.3. Statistical analyses

Statistical analyses were carried out using SPSS version 17.0 (IBM, USA) and GraphPad Prism™ version 5.00 (GraphPad Software, Inc., CA, USA) software. P-values of less than 0.05 were considered significant. Numerical variables have been summarized by their median and interquartile range and categorical variables by number of patients and percentage (%) of each category. Allele burden results were presented as the median of percentage of mutated alleles, and groups were compared by Kruskal–Wallis test. Mann–Whitney test was used for continuous variables, while for categorical variables Chi-square and Fisher's exact tests were used.

3. Results

Among the 65 studied patients, 36 (55.4%) presented *JAK2*^{V617F}, one (1.5%) *MPL*^{W515L}, and 18 (27.7%) *CALR* mutations. Ten patients (15.4%) were considered triple negative, as they lacked *JAK2*^{V617F}, *MPL*$^{W515K/L}$ or *CALR* mutations. The mutation frequencies in each studied group are described in Fig. 1.

The median of allele burden for *JAK2*^{V617F} was 25.5% in PMF, 9.0% in ET and 9.9% in MPET patients, and the differences were not significant ($p = 0.351$). Two *JAK2*^{V617F} positive patients also presented karyotype alterations, one PMF (46,XX, del(20)(q4q13)[2]/46,XX[15]) and one MPET patient (46,XY,del(20)(q11.2)[5]). Both of them had higher allele burden percentages than the median of their respective groups (79.7% and 70.8%, respectively).

Among 29 WT *JAK2*^{V617F} and *MPL*$^{W515K/L}$ patients, 18 (62.1%) presented *CALR* mutations: 80.0% (8/10) of those with PMF, 77.8% (7/9) of those with MPET, and 30.0% (3/10) of those with ET. The most frequent *CALR* mutation in all patients were type 1 (p.L367fs*46), followed by type 2 (p.K385fs*47). There were nine (32.1%) type 1 and six (21.4%) type 2 mutations, while the remaining included type 32 (p.K385fs*46) and type 40 (p.K375fd*49), as well as a novel mutation (*1125delA*). ET patients did not harbor type 2 mutation. The group distribution of *CARL* mutations are showed in Table 1.

All the samples showed concordance between fragment and sequencing analysis of *CALR* exon 9 mutations, except for the PMF patient harboring the novel mutation (*1125delA*). The misidentification of a single base deletion in the fragment analysis can be attributed to its technical limitation to recognize small insertions and deletions. This patient did not show any different clinical or hematological data than patients harboring type 1 or type 2 mutations ($p > 0.05$). This 61-year-old man had severe anemia (hemoglobin level, 5.8 g/dL), a leukocyte count of 4.200/mm^3 and platelet count of 133.000/mm^3. He did not present thrombotic events or diabetes, but he was hypertensive. The novel mutation *1125delA* leads to a frameshift that changes the secondary structure of the protein resulting in an alpha-helix conformation.

Clinical and hematological features of 64 patients stratified by their mutation profile (*CALR* mutated, *JAK2*^{V617F} and triple negative) are listed in Table 2. *MPL*^{W515L} positive patient was not included in these analyses. Patients carrying *CALR* mutations had higher platelet count ($p = 0.032$) and lower splenomegaly frequency ($p = 0.030$) than *JAK2*^{V617F}, while triple negative patients had higher body mass index than *JAK2*^{V617F} carriers ($p = 0.011$). Furthermore, triple negative patients also had higher C-reactive protein levels ($p = 0.011$) than patients carrying *CARL* mutations.

PMF *CALR* mutant carriers showed lower red blood cell count ($p = 0.048$), hemoglobin levels ($p = 0.021$), hematocrit ($p = 0.021$), C-reactive protein levels ($p = 0.035$) and less presence of splenomegaly ($p = 0.031$) than *JAK2*^{V617F} carriers. Furthermore, PMF *CALR* mutated patients showed lower frequency of thrombotic events ($p = 0.019$) in comparison with *JAK2*^{V617F} carriers, while C-reactive protein levels