Impact of Calreticulin Mutations on Clinical and Hematological Phenotype and Outcome in Essential Thrombocythemia

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*, equal contribution

Running head: CALR Mutations in Essential Thrombocythemia

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Key points

- CALR mutations occur in half of JAK2 and MPL wild type patients with ET and associate with some distinctive phenotypic traits
- Patients with ET harboring CALR mutations are at significantly lower risk of thrombosis compared with JAK2 and MPL mutated

Abstract

Mutations in the calreticulin (CALR) gene were recently discovered in patients with essential thrombocythemia (ET) lacking the JAK2V617F and MPLW515 mutations, but no information is available on the clinical correlates. In this series, CALR mutations were found in 15.5% of 576 WHO-defined ET subjects accounting for 48.9% of JAK2 and MPL wild type. CALR mutated patients were preferentially male and showed higher platelet count, lower hemoglobin and leukocyte count compared with JAK2 and MPL mutated patients. Subjects carrying CALR mutation had a lower risk of thrombosis than JAK2 and MPL mutated; of interest, their risk was superimposable to patients who were wild type for the above mutations. CALR mutation had no impact on survival or transformation to post-ET myelofibrosis. Genotyping for CALR mutations represents a novel useful tool for establishing a clonal myeloproliferative disorder in JAK2 and MPL wild type patients with thrombocytosis and may have prognostic and therapeutic relevance.
Introduction

Unlike polycythemia vera, where virtually all the patients harbor JAK2 mutations (JAK2V617F in >95% and JAK2 exon 12 mutations in about 2-4%), only 50-60% of essential thrombocytemia (ET)\(^1\)–\(^3\) and primary myelofibrosis (PMF)\(^4\)–\(^6\) patients are JAK2V617F mutated (JAK2\(^+\)). An additional 3-5% of ET and PMF subjects present mutations of MPL at codon 515 (MPL\(^+\))\(^7\)–\(^9\). JAK2 and MPL mutations represent major diagnostic criteria in the 2008 WHO classification of chronic myeloproliferative neoplasms (MPN)\(^10\),\(^11\). All these mutations result in the abnormal activation of the JAK/STAT signaling pathway, that represents an hallmark of MPN and a target for therapy\(^12\). Recently, mutations at exon 9 of CALR, the gene encoding calreticulin, an endoplasmic reticulum Ca\(^{2+}\) binding chaperone, were discovered in 50-70% of patients with ET and PMF (CALR\(^+\)) who were wild type (wt) for JAK2 and MPL\(^13\),\(^14\). The mechanisms by which CALR mutations produce a myeloproliferative phenotype are unknown.

The aim of this study was to describe the prevalence, characteristics and clinical and laboratory features associated with CALR mutations in a large population of patients with WHO-defined ET.

Methods

The study involved 576 patients with a diagnosis of ET fulfilling the 2008 WHO criteria who were followed at the Hematology Department, University of Florence. They had a stored sample of granulocyte DNA collected at diagnosis or within 3 years. Patients had provided an informed written consent in accordance with the Declaration of Helsinki for the use of left DNA for investigational purposes. The ethical committee was that of the Azienda ospedaliero-universitaria Careggi in Florence. The JAK2V617F and MPLW515L/K mutation were assessed by Real Time quantitative
PCR\textsuperscript{15,16} and for MPL also by high-resolution melting (HRM) analysis followed by bidirectional Sanger sequencing\textsuperscript{17}. Mutations in exon 9 of CALR were assessed by bidirectional sequencing\textsuperscript{13}.

Patients’ characteristics reported in Table 1 were obtained at diagnosis. Splenomegaly was defined as a palpable organ below the left costal margin. Major thrombosis and bleeding, at diagnosis and/or in the two preceding years and/or anytime during the follow-up, were recorded when objectively documented\textsuperscript{18} and according to standard definitions\textsuperscript{19}. Microvessel symptoms consisted of erythromelalgia, recurrent episodes of otherwise unexplained blurred vision, tinnitus, paresthesia, headache. Constitutional symptoms included fever, unintentional weight loss, night sweats. Pruritus was recorded when described as a diffuse, recurrent itching exacerbated by water contact. Evolution to post-ET myelofibrosis (PET-MF) and acute leukemia (AL) was diagnosed following the IWG-MRT and WHO criteria, respectively\textsuperscript{11,20}. Patients were treated according to current recommendations\textsuperscript{21}; cytoreduction was hydroxyurea in >90% of high-risk patients.

Statistical analysis was performed with SPSS software. Patients’ characteristics were compared with the use of chi-square test or Fisher’s exact test for categorical variables and t-test or nonparametric test for continuous variables. Significance level was p<0.05 in two-sided tests. Survival estimates were obtained with Kaplan-Meier method; the hazard ratio (HR) was determined using Cox proportional hazards model.

Results and Discussion

In the entire patient series, the median age was 58.1 y (range, 13-93); 194 subjects (33.7%) were male. Median follow-up was 71.9 months (2-257); seventy patients (12.1%) had died after a median of 53.9 months (2-249) from diagnosis. Nineteen patients (3.3%) progressed to PET-MF after a
median of 122 months (19-248); transformation to PV was documented in 5 cases (0.9%) and four patients (0.7%) evolved to AL after a median of 117.7 months (56-250). We found 89 subjects (15.5% of total) harboring exon 9 CALR mutations. CALR mutations were represented by insertions and deletions, as reported\textsuperscript{13,34}. Deletions (60.7%) occurred more frequently than insertions (39.3%); the commonest deletion was del367fs46 (37.0%) and ins385fs47 (71.4%) among insertions. JAK2V617F and MPLW515 mutations occurred in 64.1% (n=369) and 4.3% (n=25) of the patients. CALR\textsuperscript{*} patients accounted for 48.9% of JAK2 and MPL wt patients (n=182); 93 subjects (16.1% of total) were wt for the three mutations considered.

We compared hematological and clinical characteristics of the patients who were categorized according to their JAK2V617F, MPLW515 and CALR genotype (Table 1). CALR\textsuperscript{*} patients were younger than JAK2\textsuperscript{*} and no different from MPL\textsuperscript{*} and wt; a striking male predominance was found among CALR\textsuperscript{*} (59.5%) compared with JAK2\textsuperscript{*} (31.7%; p<0.001), MPL\textsuperscript{*} (24.0%, p=0.002) and wt (19.4%; p<0.001) patients. Influence of gender on JAK2V617F allele burden\textsuperscript{22}, disease class and vascular complications\textsuperscript{23} is well documented, and current data add to the understanding of the role of host variations for the expression of the MPN phenotype\textsuperscript{24}.

The leukocyte count, the hemoglobin and the hematocrit level were lower in CALR\textsuperscript{*} compared with JAK2\textsuperscript{*} (p=0.001, p<0.0001 and p<0.0001, respectively) and similar to MPL\textsuperscript{*} and wt subjects; on the other hand, platelet count was higher in CALR\textsuperscript{*} than in JAK2\textsuperscript{*} and wt subjects (p<0.0001 for both), but comparable to MPL\textsuperscript{*} patients who also differed significantly from JAK2\textsuperscript{*} (p<0.001). Lactate dehydrogenase level (LDH) was lower in wt compared to patients with any mutation (p<0.001). Constitutional symptoms and pruritus were similarly represented in the different groups; a palpable spleen was less common in wt subjects compared to those harboring any mutation (p<0.01).
proportion of \( CALR^+ \) patients who received cytoreduction was similar to \( JAK2^+ \) and \( MPL^+ \) and lower than wt (Table 1). Overall, these findings indicate that \( CALR^+ \) patients, similar to \( MPL^+ \), present a phenotype associated with preferential expansion of the megakaryocytic lineage compared with favored erythropoiesis in \( JAK2^+ \) subjects.

Major cardiovascular events occurred in 30.1%, 40.0%, 13.5% and 16.1% of the \( JAK2^+ \), \( MPL^+ \), \( CALR^+ \) and wt subjects; the difference was statistically significant when comparing \( CALR^+ \) versus \( JAK2^+ \) and \( MPL^+ \) subjects (p=0.01 for both). On the other hand, microvessel symptoms were more represented among \( MPL^+ \) patients (p<0.01 compared to the other groups). The thrombosis free survival was significantly longer in \( CALR^+ \) and wt subjects compared to \( JAK2^+ \) and \( MPL^+ \) (p=0.008) (Figure 1A). The cumulative incidence of thrombosis at 10 years was 5.12% (95%CI, 1.6-15.2) in \( CALR^+ \), 14.54% (95%CI, 10.0-20.8) in \( JAK2^+ \), 19.46% (95%CI, 7.6-44.6) in \( MPL^+ \), and 8.17% (95%CI, 2.7-23.3) in wt subjects. Taking wt subjects as reference population, the hazard ratio (HR) for thrombosis was 0.74 (95%CI, 0.33-1.00) for \( CALR^+ \), 1.78 (95%CI, 1.06-3.18) for \( JAK2^+ \) and 1.65 (95%CI, 1.7-3.92) for \( MPL^+ \) patients. There was a trend towards more frequent hemorrhages in \( MPL^+ \) compared to all other patients. The median survival was not reached in any group, and Kaplan Meier estimates of survival did not show significant differences (Figure 1B). \( CALR^+ \) patients were preferentially distributed in the lower risk category of the Thrombosis score, IPSET-thrombosis and IPSET score compared with \( JAK2^+ \) (and \( MPL^+ \) for IPSET-thrombosis) (Supplemental Table 1). Overall, these data indicate that \( CALR^+ \) patients are less prone to thrombotic events compared to \( JAK2^+ \) and \( MPL^+ \); of note, their risk was similar to subjects lacking any mutations.

Transformation to PET-MF occurred in 19 patients; the HR for PET-MF was similarly increased in \( CALR^+ \) (2.36; 95%CI, 0.26-21.8), \( JAK2^+ \) (2.21; 95%CI, 0.28-17.8) and \( MPL^+ \) (2.50; 95%CI, 0.22-28.5)
patients compared with wt, although possibly due to small number of events did not reach the significance level. Noteworthy, all 5 cases of transformation to PV occurred in the JAK2* patients.

With the limitations imposed by its observational nature, that precludes any causal relationships inferences, results of current study identified meaningful associations between the presence of CALR mutations and the phenotype of patients with ET. The findings that CALR mutated patients are at lower risk of vascular events may have implications for risk stratification and management. Finally, our study underscores the importance of CALR genotyping for an accurate diagnosis of patients with thrombocytosis who lack the JAK2V617F and MPLW515 mutation.
Acknowledgments

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Authorship Contributions

PG, AMV: designed the study, analyzed the data, and wrote the manuscript; GR, CM, performed molecular analysis, analyzed raw sequencing data; AP, AP, TF contributed to molecular analysis; PG, LP, AB, AMV contributed samples and clinical information. All authors have read the final version of the manuscript and agreed on its content.

Disclosure of Conflict of Interest

There is no conflict of interest to disclose for the work performed in this manuscript.
References


**Table 1.** Laboratory and clinical characteristics of CALR mutant patients compared to JAK2V617F or MPLW515 mutant patients and subjects who were wild type for the above three mutations.

<table>
<thead>
<tr>
<th></th>
<th>CALR(^+) (A)</th>
<th>JAK2 V617F(^+) (B)</th>
<th>MPL W515(^+) (C)</th>
<th>CALR, JAK2, MPL wt (D)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>89 (15.5)</td>
<td>369 (64.1)</td>
<td>25 (4.3)</td>
<td>93 (16.1)</td>
<td></td>
</tr>
<tr>
<td>Male no-(%)</td>
<td>53 (59.5)</td>
<td>117 (31.7)</td>
<td>6 (24.0)</td>
<td>18 (19.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age, years</td>
<td>54.7 (13-88)</td>
<td>61 (15-93)</td>
<td>54 (22.89)</td>
<td>53 (15-87)</td>
<td>0.04</td>
</tr>
<tr>
<td>Leukocyte count (x10(^9)/L)</td>
<td>8.1 (3.5-26.0)</td>
<td>8.9 (4.2-35.0)</td>
<td>8.4 (4.5-16.6)</td>
<td>8.3 (4-16.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>138 (106-173)</td>
<td>145 (102-173)</td>
<td>136 (110-160)</td>
<td>136 (106-164)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelet count (x10(^9)/L)</td>
<td>866 (504-2348)</td>
<td>726 (455-1881)</td>
<td>898 (607-2000)</td>
<td>697 (482-1659)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>320 (142-725)</td>
<td>288 (102-1178)</td>
<td>365 (254-570)</td>
<td>268 (137-554)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Splenomegaly, no-(%)</td>
<td>24 (27.0)</td>
<td>91 (24.7)</td>
<td>9 (36.0)</td>
<td>9 (9.7)</td>
<td>0.661</td>
</tr>
<tr>
<td>Pruritus, no-(%)</td>
<td>5 (5.6)</td>
<td>32 (8.7)</td>
<td>1 (4.0)</td>
<td>9 (9.7)</td>
<td>0.260</td>
</tr>
<tr>
<td>Constitutional symptoms, no-(%)</td>
<td>1 (1.1)</td>
<td>19 (5.1)</td>
<td>1 (1.2)</td>
<td>6 (6.5)</td>
<td>0.120</td>
</tr>
<tr>
<td>Major thrombosis, no-(%)</td>
<td>12 (13.5)</td>
<td>111 (30.1)</td>
<td>10 (40.0)</td>
<td>15 (16.1)</td>
<td>0.011</td>
</tr>
<tr>
<td>Microvessel symptoms, no-(%)</td>
<td>22 (24.7)</td>
<td>101 (27.4)</td>
<td>14 (56.0)</td>
<td>20 (21.5)</td>
<td>0.604</td>
</tr>
<tr>
<td>Major hemorrhage, no-(%)</td>
<td>4 (4.5)</td>
<td>17 (4.6)</td>
<td>4 (16.0)</td>
<td>3 (3.3)</td>
<td>0.906</td>
</tr>
<tr>
<td>Progression to PET-MF, no-(%)</td>
<td>4 (4.5)</td>
<td>12 (3.3)</td>
<td>2 (8.0)</td>
<td>1 (1.1)</td>
<td>0.458</td>
</tr>
<tr>
<td>Progression to PV, no-(%)</td>
<td>0</td>
<td>5 (1.4)</td>
<td>0</td>
<td>0</td>
<td>0.294</td>
</tr>
<tr>
<td>Progression to AL, no-(%)</td>
<td>0</td>
<td>2 (0.5)</td>
<td>1 (4.0)</td>
<td>1 (1.1)</td>
<td>0.507</td>
</tr>
<tr>
<td>Deceased (n=70; %)</td>
<td>10 (11.2)</td>
<td>49 (13.3)</td>
<td>4 (16.0)</td>
<td>7 (7.5)</td>
<td>0.598</td>
</tr>
<tr>
<td>Cytoreductive therapy, no-(%)</td>
<td>50 (62.5)</td>
<td>220 (60.8)</td>
<td>19 (76.0)</td>
<td>40 (46.0)</td>
<td>0.440</td>
</tr>
</tbody>
</table>

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Hematologic and clinical information were collected at diagnosis; information regarding major thrombosis and hemorrhage included events at diagnosis, in the two preceding years and during the follow-up. Cytoreduction means that the patient received cytoreductive drugs (in >90% of cases, hydroxyurea) during the course of disease at the physician discretion, based on conventional criteria. Unless otherwise indicated, values are reported as median (range).

wt= wild type; PET-MF post-essential thrombocytemia myelofibrosis; PV= polycythemia vera; AL= acute leukemia.
Figure legend.

**Figure 1.** Kaplan Meier estimate of thrombosis-free survival (panel A) and overall survival (panel B) in patients who were categorized according to their mutational status (**JAK2V617F**, **MPLW515K**, **CALR**<sup>+</sup>, or wild type for the above mutations).
Figure 1
Impact of Calreticulin Mutations on Clinical and Hematological Phenotype and Outcome in Essential Thrombocythemia

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