CHARACTERIZATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS
IN JAK2V617F AND CALR-MUTATED MYELOPROLIFERATIVE
NEOPLASMS

Running title: JAK2V617F and CALR-mutated stem cells in myeloproliferative neoplasms

Anna Angona, Alberto Alvarez-Larrán, Beatriz Bellosillo, Raquel Longarón, Laura Camacho, M. Concepción Fernández-Rodríguez, Silvia Pairet, Carles Besses

1 Hematology Department, Hospital del Mar, Barcelona, Spain. 2 Pathology Department, Hospital del Mar, Barcelona, Spain. 3 Universitat Autònoma de Barcelona, Barcelona, Spain. 4 Universitat Pompeu Fabra, Barcelona, Spain. 5 Grup de Recerca Clínica Aplicada en Neoplàsies Hematològiques-Hospital del Mar-IMIM, Barcelona, Spain.

Correspondence: Alberto Alvarez-Larrán, MD, Hematology Department, Hospital del Mar, Passeig Marítim 25-29, 08003 Barcelona, Spain. Phone: +34 932483341. Fax: +34 932483343. E-mail: 95967@parcdesalutmar.cat
Abstract

Mutations in JAK2 or CALR are observed in patients with myeloproliferative neoplasms (MPN). To get further insight in the dynamics of the mutant clone, we assessed the mutant allele burden in hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and granulocytes from 138 patients [51 polycythemia vera (PV), 58 essential thrombocythemia (ET) and 29 myelofibrosis (MF)]. CALR-mutated ET patients harbored a higher mutant load at progenitor level than JAK2V617F-positive ET (HSCs: 39.9% vs 7.5% p< 0.001, HPCs: 32.7% vs 7.7% p< 0.001). Moreover, HSCs of CALR-mutated ET patients showed a similar mutational load than patients with CALR-mutated MF (39.9% vs 48.2%, p=0.17). Regarding JAK2V617F MPN, PV and ET patients showed a low mutational burden at progenitor level whereas in the myelofibrotic phase the dominance of the mutated clone was a constant finding. In conclusion, the size of the mutated clone in chronic phase MPN is different according to genotype with CALR-mutated ET showing a pattern similar to that observed in MF.
**Keywords:** myeloproliferative neoplasms, \textit{JAK2V617F}, \textit{CALR}, allele burden, hematopoietic stem cells

**Introduction**

Polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF) are myeloproliferative neoplasms (MPN) characterized by overproduction of one or more mature myeloid cell types. The origin of these neoplasms is in hematopoietic stem cells (HSCs) which display self-renewal capacity and ability to differentiate into more mature myeloid cells.

The \textit{JAK2V617F} mutation is detectable in more than 95% of patients with PV and in 50-60% of those with ET or MF [1-4]. In the chronic phase of PV or ET, \textit{JAK2V617F} is present in a low range at progenitor level with the percentage of CD34+ cells harboring \textit{JAK2V617F} increasing at a low rate during the follow-up of the disease [5-9]. A constant finding in \textit{JAK2V617F}-mutated PV and ET is the existence of a higher allele burden in granulocytes than in progenitors suggesting that mutated progenitors have a greater differentiation capacity than wild-type stem cells [5]. In contrast, in primary and secondary MF, \textit{JAK2V617F} allele burden is very high in both granulocytes and progenitor cells indicating that the dominance of the \textit{JAK2V617F}-positive clone at the CD34+ compartment is an important modifier of the disease phenotype in \textit{JAK2V617F}-positive MPNs [10].

Recently, somatic mutations at exon 9 \textit{CALR}, the gene encoding calreticulin, have been described in around 40-70% of \textit{JAK2V617F} and \textit{MPL} wild-type ET and MF patients [11, 12]. Many types of \textit{CALR} mutations have been reported, but the most frequent alterations are the 52-bp deletion (mutation type 1) and the 5-bp insertion (mutation type 2). The mechanisms by
which CALR mutants causes MPN are still under investigation, but recent data show that they activate the thrombopoietin receptor and in turn JAK2/STAT pathway [13]. Interestingly, the mutant allele burden in granulocytes is higher in CALR-mutated ET than in JAK2V617F-mutated ET [14]. However, there is limited information regarding the size of the CALR mutant clone in the progenitor compartment.

The aim of this work was to study the mutant allele burden at the progenitor level in CALR-mutated MPN and compare it to the corresponding in JAK2V617F-positive progenitor cells.

Patients and methods

Patients

From a whole cohort of 395 JAK2V617F or CALR-mutated MPN, a total of 138 patients were included in the study. Overall, we studied 51 PV (all JAK2V617F positive), 58 ET (35 JAK2V617F and 23 CALR-mutated) and 29 MF (20 JAK2V617F and 9 CALR-mutated). Regarding JAK2V617F-mutated MF, 7 were primary MF, 11 post-PV MF and 2 post-ET MF. Additionally, 4 of CALR-mutated MF were primary MF and the others were post-ET MF. At the time of the study, 104 patients were in early chronic phase (< 5 years from diagnosis) and 34 patients in late chronic phase (more than 5 years from diagnosis). Patients were diagnosed according to World Health Organization criteria [15]. Patients with the pre-fibrotic form of MF were excluded. Informed consent for the scientific use of the patients’ clinic hematological data and
biological samples was obtained, and the study was approved by the Hospital del Mar Ethics Committee.

Isolation of granulocytes and CD34+ cells

Granulocytes were isolated from peripheral blood or blood marrow samples by density gradient, whereas CD34+ cells were purified by immunomagnetic positive selection (Miltenyi Biotech, Bergisch Gladbach, Germany) from the mononuclear cell layer obtained after Ficoll centrifugation. Stem cells CD34+CD38- (HSCs) and progenitor cells CD34+CD38+ (HPCs) were further separated by fluorescence-activated cell sorting using a Becton Dickinson sorter (BD Biosciences, San Jose, CA, USA).

JAK2V617F and CALR mutation allele burden quantification

JAK2V617F allele burden was assessed in DNA from HSCs, HPCs and granulocytes by quantitative allele specific polymerase chain reaction [16]. The mutational analysis of exon 9 of the CALR gene was performed in the three populations by PCR, using a 6-carboxyfluorescein labelled reverse primer, followed by fragment analysis in a Genetic Analyzer 3500DX (Applied Biosystems).

Statistical methods

The mutant allele load in the different cell populations (HSCs, HPCs and granulocytes) was compared according to disease type (JAK2V617F PV, JAK2V617F ET, CALR-mutated ET, JAK2V617F MF and CALR-mutated MF).
Differences between groups were analyzed with t-Student test or one-way ANOVA followed by post-hoc analysis using the Student-Newman-Keuls test for continuous variables and the Chi-square test for categorical variables. Significance was considered for p values <0.05. The statistical analysis was performed using the SPSS 21.0 package (SPSS, Chicago, IL).

Results

Patients’ characteristics

The main clinical and hematological data at the time of the study of the 138 MPN patients are summarized in Table 1. At the moment of CD34+ cell purification, patients with MF and those with CALR-mutated ET were more frequently on cytoreductive therapy than patients with PV or JAK2V617F-mutated ET. Type 1, type 2 and other types of CALR mutations were detected in 16 (ET n=8, MF n=8), 11 (ET n=11) and 5 (ET n=4, MF n=1) cases, respectively.

Mutant allele burden in HCSs, HPCs and granulocytes according to the disease

The quantification of JAK2V617F or CALR mutation allele burden in each population according to the disease is shown in Figure 1.

The mutant allele burden was significantly lower in JAK2V617F ET than in JAK2V617F PV in the three cell populations studied: HSCs (7.5% vs 16.7%, p =0.002) HPCs (7.7% vs 26.9%, p < 0.001) and granulocytes (21% vs 62.9%, p < 0.001) (Figure 2a).

As can be seen in Figure 2b, CALR-mutated ET patients harbored a higher mutant load at progenitor level than JAK2V617F-positive ET (HSCs: 39.9% vs 7.5% p< 0.001, HPCs: 32.7% vs 7.7% p< 0.001). These differences
were also observed in granulocytes (35.1% vs 21% p< 0.001). In addition, the
comparison of CALR-mutated ET with CALR-mutated MF showed no
statistically significant differences in the mutant allele burden of stem cells
(39.8% vs 48.2%, p=0.17) and progenitors (32.7% vs 47.7%, p=0.09) (Figure
2c). Similar results were observed when patients with early or late chronic
phase were separately considered.

Regarding CALR-mutated ET patients, no significant differences
according to the type of the mutation were found neither at progenitor level nor
in granulocytes (type 1 in comparison with type 2: HSCs 43.2% vs 36.7%
p=0.54, HPCs 41.2% vs 25.9% p=0.16, granulocytes 35.6% vs 37.1% p =0.8)
(Figure 2d).

Patients with MF showed the highest mutant load at the progenitor level
in comparison with the remaining groups. Taking into consideration the
mutational status of MF, JAK2V617F-MF patients showed a significantly higher
mutant load in granulocytes than those with mutation in CALR gene (66.4% vs
51.4%, p =0.04), whereas no significant differences were observed at the
progenitor level (Figure 2e).

The mutant allele burden in each population was compared between
patients who were under cytoreductive treatment at the moment of CD34+
selection and those who were free of therapy. No significant differences were
observed nor in PV, ET (JAK2V617F and CALR-mutated) neither CALR-
mutated MF. However, in JAK2V617F MF, the mutant load was significantly
higher in the three cell populations in patients who were receiving cytoreductive
therapy in contrast to those without treatment (data not shown).
Dynamics of mutant allele burden during the evolution of the disease

We compared the mutant allele burden in each population according to the evolutive phase of the disease (early chronic phase or late chronic phase) (Table 2). As can be seen patients with shorter disease duration harbored a significantly lower allele burden in the three populations than patients with more follow-up. The increase in the mutant allele burden at progenitor level was higher in CALR-mutated than in JAK2V617F-mutated MPN (Table 2). We analyzed CALR-mutated ET patients according to type of the mutation, and in both cases the mutant allele burden increased in all populations during the follow-up (data not shown).

Discussion

Around 90% of MPN patients carry at least one somatic mutation, being JAK2V617F the most frequent (69%), followed by mutations in exon 9 of CALR gene (15%) [17]. In this study we assessed the mutant allele burden in HSCs, HPCs and granulocytes of 138 MPN patients. The main findings were a significantly higher mutant allele burden at progenitor level in CALR-mutated ET than in JAK2V617F ET being the CALR mutant allele load in ET similar to the one observed in patients with MF.

It has been proposed that JAK2V617F-mutated ET and PV represent a biological spectrum of the same disease modulated by several acquired and constitutional factors [18]. JAK2V617F-homozygous erythroid progenitors have
been reported to occur in most patients with PV but rarely in those with ET [19]. Moreover, Godfrey et al, using microsatellite PCR to map loss-of-heterozygosity in colony assays, have observed that PV is associated with expansion of a dominant homozygous clone [20]. Furthermore, other factors have been involved in the acquisition of a particular phenotype such as the polymorphism HBS1L-MYB (rs9376092) [21], a defect in STAT1 phosphorylation [22] and the order of acquisition of additional mutations [23]. In our study we observed that the mutant load at the progenitor level in JAK2V617F mutated patients increases in accordance to the phenotype, being higher in JAK2V617F PV than in JAK2V617F ET. This finding is in accordance with more frequent homozygous cells observed in colonies derived from PV patients [20]. When JAK2V617F MF patients were analyzed, the mutant load was higher than 50% in the majority of patients indicating that JAK2V617F clonal dominance is a constant finding in these patients, as previously reported [7, 10]. All this data support the idea that JAK2V617F ET and JAK2V617F PV represent a continuum of the same disease and MF transformation is a late event during the follow-up.

Recently, distinct phenotype and clinical characteristics have been described between JAK2V617F and CALR-mutated ET. Thus, mutation in CALR gene is associated with younger age, lower level of hemoglobin and leukocytes, and higher platelet count, and interestingly, a lower risk of thrombosis in comparison with JAK2-mutated patients [14, 24]. In addition to this different clinical profile, several studies argue that JAK2V617F and CALR mutations define two distinct diseases at the molecular level. In this sense, X chromosome inactivation patterns (XCIP) analyses showed clonality in 88% of
CALR-mutated ET patients in comparison with 26% of JAK2V617F ET patients [25]. Moreover, some studies suggest that mutation acquisition is a late event in JAK2V617F-positive MPN whereas CALR mutations are typically initiating events [11, 12, 17].

Moreover, it is important to note that CALR-mutated ET patients have a higher mutant load at the progenitor level than JAK2V617F-positive ET and JAK2V617F PV patients, conferring a higher potential to proliferate during the chronic phase of MPN. Interestingly, when we compared CALR-mutated ET and CALR-mutated MF, no significant differences in the mutant allele burden at the progenitor level were observed. The existence of an expanded mutated clone at progenitor level in CALR-positive ET might indicate that MF and ET are a continuum of the same disease. In this sense, it has been reported a higher frequency of MF transformation in patients with CALR-mutated ET than in those with JAK2V617F [12, 26]. In addition, CALR-mutated ET showed at diagnosis some MF-like clinical features such as lower hemoglobin [14, 24, 26-28], or higher LDH serum level [28] than JAK2V617F ET. Finally, bone marrow histology is different according to genotype with CALR-positive ET being more frequently associated with megakaryocyte abnormalities and prefibrotic PMF [29]. In this sense, it would be interesting to study the allele burden at the stem cell level in patients with pre-fibrotic form of MF according to genotype. It has been reported that ET patients with type 1 CALR mutation showed a higher risk of MF transformation compared with type 2 or other mutations [30]. We could not demonstrate differences in the mutant allele burden of progenitor cells according to the type of CALR mutation in ET. However, this finding must be interpreted cautiously due to the limited number of cases.
Regarding MF, we could observe the presence of clonal dominance in the majority of JAK2V617F-positive cases as previously described [7, 10], and the same was observed in CALR-mutated MF. However, while JAK2V617F allele burden showed a slight rise between HSCs and granulocytes, the mutant allele burden in those patients harboring CALR was similar in the three populations, suggesting higher differentiation potential of JAK2V617F.

We observed that JAK2V617F allele burden increased steadily during the hematopoiesis process in PV and, in a lower range, also in ET. However, when we analyzed CALR-mutated ET, the mutant load remained stable in HSCs, HPCs and granulocytes. These results support the fact that JAK2V617F confers a higher differentiation potential than CALR mutation. In this regard, it has been reported a higher frequency of monoclonal hematopoiesis and suppression of wild-type myeloid cells at the hematopoietic stage in the CALR-mutated ET but not in JAK2V617F ET [25].

Finally, our work provides data concerning the changes of the mutant allele burden during the evolution of the disease. Thus, we have shown that the mutant allele burden increases significantly in the three populations both in JAK2V617F and CALR-mutated MPN. Interestingly, in CALR-mutated MPN, the clonal dominance remained stable during the evolution of the disease, suggesting a continuous proliferation of the mutated clones.

One of the limitations of this study is the scarce number of patients analyzed. Another important limitation is that we used only CD38 and CD34 staining to define HSCs and HPCs in contrast to other researchers that have added CD90 and CD45RA to improve the discrimination between HSCs and HPCs [31]. However, taking into consideration that CD38 positivity showed a
continuous gradient, the double CD38intermediate+/CD34+ population was excluded in order to be sure that HSCs and HPCs were CD38- and CD38+ positive, respectively. Finally, we assumed the mutated clone is embedded in an environment of non-mutated cells that maintain a normal proliferative and differentiate capacity. However, the cross-talks of malignant cells with the microenvironment and residual normal haematopoiesis may be different according to genotype.

In conclusion, the size of the mutated clone at the stem cell level is different according to the genotype of MPN. CALR-mutated MPN are characterized by an expansion of mutated progenitors with suppression of wild type mature cells whereas JAK2V617F-mutated PV and ET have a low proportion of mutated progenitors with an enhanced ability of differentiation.
**Authorship**

A.Ang. designed the study, collected the data, performed the molecular studies, performed the statistical analysis, analyzed and interpreted the results and wrote the paper. A.A.L. designed the study, collected the data, performed the statistical analysis, analyzed and interpreted the results and wrote the paper. B.B. and C.B. designed the study, interpreted the results, wrote the paper and approved the final version. R.L., L.C., S.P. and C.F.R. performed the molecular studies and approved the final version.

No relevant conflict of interest to declare regarding this article.
References


Table 1. Main clinical and hematological characteristics at the time of CD34+ cells purification in 138 MPN patients included in the study

<table>
<thead>
<tr>
<th></th>
<th>PV  N = 51</th>
<th>TE JAK2V617F N = 35</th>
<th>TE CALR- mut N = 23</th>
<th>MF JAK2V617F N = 20</th>
<th>MF CALR-mut N = 9</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time from diagnosis, months^1</td>
<td>1 (0-271)</td>
<td>0 (0-179)</td>
<td>51 (0-269)</td>
<td>7 (0-131)</td>
<td>8 (0-223)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Early/late chronic phase, n</td>
<td>36/15</td>
<td>34/1</td>
<td>12/11</td>
<td>16/4</td>
<td>6/3</td>
<td>0.002</td>
</tr>
<tr>
<td>Age, years^1</td>
<td>68 (41-95)</td>
<td>65 (31-85)</td>
<td>66 (32-93)</td>
<td>72 (51-85)</td>
<td>72 (35-90)</td>
<td>0.56</td>
</tr>
<tr>
<td>Male/female, n</td>
<td>35/16</td>
<td>13/22</td>
<td>9/14</td>
<td>8/12</td>
<td>4/5</td>
<td>0.02</td>
</tr>
<tr>
<td>Hemoglobin, g/L^1</td>
<td>161 (106-222)</td>
<td>147 (124-169)</td>
<td>125 (95-153)</td>
<td>109 (91-146)</td>
<td>110 (66-140)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Leukocytes, x10^9/L^1</td>
<td>11.7 (5-21.1)</td>
<td>8.6 (5.8-16.4)</td>
<td>7.4 (3.4-10.9)</td>
<td>9.9 (3.4-48.4)</td>
<td>9.3 (2.8-51.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Platelets, x10^9/L^1</td>
<td>558 (160-1138)</td>
<td>617 (450-876)</td>
<td>623 (294-1149)</td>
<td>192 (44-770)</td>
<td>443 (43-645)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cytoreductive treatment, n (%)</td>
<td>16 (31.4)</td>
<td>2 (5.7)</td>
<td>10 (43.5)</td>
<td>13 (65)</td>
<td>6 (66.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>13</td>
<td>1</td>
<td>7^#</td>
<td>10##</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Mutant allele burden in HSCs, HPCs and granulocytes according to the evolutive phase of the disease

<table>
<thead>
<tr>
<th></th>
<th>Early chronic phase n=86</th>
<th>Late chronic phase n=20</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JAK2V617F MPN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSCs</td>
<td>18.4 (23.6)</td>
<td>32.9 (25.9)</td>
<td>0.017</td>
</tr>
<tr>
<td>HPCs</td>
<td>22.7 (24.2)</td>
<td>39.7 (27.04)</td>
<td>0.007</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>46.2 (29.7)</td>
<td>65.1 (26.7)</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>CALR-mutated MPN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSCs</td>
<td>35.4 (17.8)</td>
<td>55.4 (10.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>HPCs</td>
<td>28.3 (20.4)</td>
<td>54.9 (9.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>33.7 (14.2)</td>
<td>46.02 (12.8)</td>
<td>0.02</td>
</tr>
</tbody>
</table>
**Table legends**

**Table 1.** Main clinical and hematological characteristics at the time of CD34+ cells purification in 138 MPN patients included in the study

MPN: myeloproliferative neoplasms; PV: polycythemia vera; TE: essential thrombocythemia. ¹ Median (range). ns: no significance. Cytoreductive treatment included hydroxiurea, busulphan, P32, anagrelide and ruxolitinib. p value compared to one way ANOVA or Chi-square test for continuous or quality variables, respectively. #In two patients, Hydroxyurea + Anagrelide; ##In one patient, Hydroxyurea + Anagrelide

**Table 2.** Mutant allele burden in HSCs, HPCs and granulocytes according to the evolutive phase of the disease

HSCs: hematopoietic stem cells; HPCs: hematopoietic progenitor cells. Results are expressed as mean (SD). Differences between groups are analyzed with t-Student test. Overall 31/106 JAK2V617F MPN and 16/32 CALR-mutated MPN (p=0.03) were under cytoreductive treatment (hydroxiurea, busulphan, P32, anagrelide and ruxolitinib)
Footnotes

**Figure 1.** Mutant allele burden in haematopoietic stem cells (HSCs), haematopoietic progenitor cells (HPCs) and granulocytes according to the disease.

PV: polycythaemia vera; ET: essential thrombocythemia; MF: myelofibrosis.

**Figure 2.** Comparison of the mutant allele burden in hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and granulocytes according to the disease. Differences between groups are analyzed with t-Student test. Significance was considered for p values <0.05.

a) JAK2V617F PV in comparison with JAK2V617F ET

b) CALR-mutated ET in comparison with JAK2V617F ET

c) CALR-mutated ET in comparison with CALR-mutated MF

d) CALR-mutated ET type 1 in comparison with CALR-mutated type 2

e) JAK2V617F MF in comparison with CALR-mutated MF

PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis