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Endogenous megakaryocytic colonies underline association between megakaryocytes and calreticulin mutations in Essential thrombocythemia.

Julie Mondet\textsuperscript{1,2}, Ji-Hye Park\textsuperscript{3}, Audrey Menard\textsuperscript{4}, Christophe Marzac\textsuperscript{5,6}, Serge Carillo\textsuperscript{7,8}, Emmanuel Pourcelot\textsuperscript{1,2}, Francois Girodon\textsuperscript{3,9}, Xenia Cabagnols\textsuperscript{10,11}, Laurence Lode\textsuperscript{4,12}, Nuria Socoro\textsuperscript{1,2}, Martine Chauvet\textsuperscript{13}, Claude-Eric Bulabois\textsuperscript{14}, Pascale Cony-Makhoul\textsuperscript{15}, Selim Corm\textsuperscript{16}, Jean-Yves Cahn\textsuperscript{1,14}, and Pascal Mossuz\textsuperscript{1,2}

\textsuperscript{1} Therex, TIMC-IMAG, CNRS Univ. Grenoble Alpes, France;
\textsuperscript{2} Laboratoire d’hématologie cellulaire, Institut de Biologie et Pathologie, CHU de Grenoble, France
\textsuperscript{3} Laboratoire d'Hématologie, CHU de Dijon, France
\textsuperscript{4} Molecular Hematology Laboratory, CHU Hôtel-Dieu, Nantes, France
\textsuperscript{5} AP-HP, Hôpital Saint-Antoine, Laboratoire d’Hématologie, Paris, France
\textsuperscript{6} INSERM, UMR_S 938, CDR Saint-Antoine, Paris, France
\textsuperscript{7} Laboratoire de cytologie clinique et cytogénétique, CHU Carémeau, Nîmes, France;
\textsuperscript{8} Institut des biomolécules Max Mousseron (IBMM), UMR CNRS 5247, Université de Montpellier, France;
\textsuperscript{9} INSERM U866, Faculté de Médecine, Dijon, France
\textsuperscript{10} UMR 1009 INSERM, Laboratory of Excellence GR-Ex, Villejuif, France
\textsuperscript{11} Gustave Roussy, UMR 1009, Villejuif, France
\textsuperscript{12} Hematology Laboratory, CHU St Eloi, Montpellier, France
\textsuperscript{13} Laboratoire d’oncophématologie, Institut de Biologie et Pathologie, CHU de Grenoble, France
\textsuperscript{14} Département d'Hématologie Clinique, CHU de Grenoble, France;
\textsuperscript{15} Centre Hospitalier Annecy-Genevois, Service d'hématologie, Pringy, France;
\textsuperscript{16} Centre Medipole de Savoie, Challes les eaux, France.

Correspondence: Julie Mondet, Institut de Biologie et Pathologie, Laboratoire d’hématologie cellulaire, CHU Grenoble CS10217 - 38043 Grenoble Cedex 09, France. Phone: international +33.476767222. E-mail: jmondet@chu-grenoble.fr
Calreticulin (CALR) mutations occur in 20% to 25% of myeloproliferative neoplasms (MPN) (1, 2). At least 40 CALR mutations have been reported to date, all located in exon 9. The most frequent CALR mutations are a 52-bp deletion (type 1) and a 5-bp insertion (type 2). Expression of type 1 CALR mutation was shown to induce constitutive activation of JAK-STAT signalling pathway in a Ba/F3 cell line with STAT5 phosphorylation leading to spontaneous growth in the absence of interleukin-3 (1). Furthermore, megakaryocyte lineage has been reported to play a major role in MPN pathophysiology (3, 4). In particular, haematopoietic colony formation independent of exogenous cytokines, including Endogenous megakaryocytic (EMC) and Endogenous Erythroid colonies (EEC) was shown to be a functional sign of clonal haematopoiesis due to deregulated signalling pathways in MPN. Altogether, these data suggest a particular link between megakaryocytic (MK) proliferation and deregulation of signalling due to driver mutations. However, to our knowledge, spontaneous growth of haematopoietic progenitors (EMC and EEC) has not been characterized in type 1 or type 2 CALR mutated patients.

In the current study, we analysed patterns of EMC and EEC according to molecular status (JAK2V617F, CALR, MPL, Triple negative) in a cohort of 302 essential thrombocythemia (ET) patients from 3 French University Hospitals [Grenoble (n=121); Dijon (n=121) and Nantes (n=60)]. The inclusion criteria were ET patients who benefited from in vitro cultures from bone marrow at diagnosis and for whom DNA was available. The JAK2V617F mutation was assessed using purified granulocytes by tetra-primer ARMS-PCR (5) or by allele-specific quantitative PCR (6). MPL mutations were screened by high resolution melt assay (7) and confirmed by sequencing. The mutational status of CALR was determined using previously described high-resolution sizing of fluorescent dye-labeled PCR amplificons of exon 9, with Sanger sequencing controls (1). To standardize EMC and EEC, cultures were performed from bone marrow samples using the same standardized collagen medium in all three centers (8, 9). Megakaryocytic colonies, composed of at least 4 MKs, were counted by microscopy after MGG staining of dry collagen dishes (9, 10). We analyzed EMC and EEC results both qualitatively: presence or not of EMC and/or EEC (so called “positive or negative EMC/EEC”) and quantitatively: number of EMC and/or EEC per $10^5$ cells plated. For statistical analysis, non-parametric tests were applied: Mann-Whitney test (for comparison of two groups) and McNemar’s test (paired data). Chi square or Fisher’s tests were used to compare nominal variables, and Spearman’s rank correlation to compare two continuous variables. A $p$ value <0.05 was considered to be statistically significant.
The mutational distribution was 50.8% (153/302) for JAK2V617F, 23% (69/302) for CALR, 5.3% (17/302) for MPL and 19% (57/302) for “triple negative”. Six patients lacked MPL and/or CALR data (1.9%). Among the 69 patients with CALR mutations, 35 (50.7%) patients harbored type 1 and 24 (34.8%) patients had type 2. Irrespective of mutational status, overall endogenous haematopoietic growth (defined by presence of EMC and/or EEC) was 58.9% (178/302). We observed significantly more EMC (56.3%; 170/302) than EEC (13.6%; 41/302) whatever the mutational status (p<0.001).

In order to determine the relationship between growth profile and genotype, we compared global spontaneous growth defined by EMC and/or EEC positivity according to mutational status. Patients harboring mutations such as JAK2V617F or CALR showed a significantly higher proportion of overall spontaneous growth (respectively 66% and 73.9%) compared to “triple negative” ones (25%, p<0.001). No difference in frequency of EMC and/or EEC between JAK2V617F and CALR was noted (p=0.24). On the contrary, MPL mutations were less frequently associated with EMC and/or EEC compared to CALR (respectively 47% and 73.9%, p=0.03).

As higher platelets counts were reported in patients with CALR mutations in comparison with JAK2V617F patients (11), we attempted to compare the proportion of EMC in these two subgroups (figure 1). EMC were more frequently observed in CALR than in JAK2V617F (respectively 73.9% and 61.4%) without reaching statistical significance (p=0.07). To strengthen the link between genotypes and megakaryocytic proliferation, we also compared the mean number of EMC depending on the molecular status. CALR mutations were associated with a significantly higher mean number of EMC compared to “triple negative” and JAK2V617F patients (respectively 9.36 (0-81.5); 1.75 (0-23.3) and 5.5 (0-53.3), p<0.01 and p=0.02). A tendency was observed between CALR and MPL but without statistical significance (p=0.07). Interestingly, concerning EEC, CALR mutated patients displayed a significantly weaker proportion of EEC positivity compared to JAK2V617F mutated patients (1.4% vs 22.8%, p<0.001). This percentage of EEC in JAK2V617F ET is consistent with previous data obtained with a serum-free assay (6, 9, 12). EEC were rare in the CALR-mutated population; only one type 2-mutated patient among 69 showed 2 EEC per 10^5 cells. A significantly higher proportion of EEC was associated with JAK2V617F compared to triple negative (p<0.001) and MPL (p<0.05).

Next, the endogenous megakaryocytic profile between types 1 and 2 CALR mutants and “variants” was compared. “Variants” have neither type 1 nor type 2 CALR mutations (n=10 patients). EMC frequencies were similar in type 1 and type 2 patients (respectively 82.8% and
70.8%, p=0.34). Proportion of EMC in “variants” was significantly lower than in type 1 mutants (50%, p=0.04) although a higher allelic burden was observed compared to type 1 (p<0.01). Among the population presenting EMC (figure 2), type 2 patients induced more EMC than type 1 (p=0.03, respectively mean₂=19.2 (2.5-81.5) vs mean₁=8.6 (1-46.6)). Interestingly, among those patients we observed a significant difference in allelic ratio between type 1 and type 2 (respectively mean₁=41.8% (5-65%) and mean₂=48.1% (30-73.9%), p=0.03).

Among the population presenting EMC, no difference in the number of EMC was observed between “variants” and either type 1 or type 2 even if “variants” patients displayed a higher allelic burden than type 1 (mean variant= 56.2% (48-80%), p<0.01) and type 2 (p<0.05). We wondered if a correlation existed between the number of EMC and allelic burden in the CALR mutated population and according to type of mutation (figure 3). Overall, no linear correlation was observed either in the whole CALR population (n=68), or in type 1 and type 2; (respectively rho=0.07, p=0.56; rho₁=-0.069, p₁=0.686 and rho₂=0.084, p₂=0.69). However, in the “variants”, a linear correlation between allelic burden and spontaneous megakaryocytic growth (rho=0.705, p<0.05) was found.

Klampfl and al showed that type 1 CALR mutation induced a cytokine independent growth of the murine Ba/F3 cell line (1). Our work confirmed this finding but in real MPN patients whether type 1 or type 2 mutated. Moreover, our results highlight a special link between CALR and megakaryocyte proliferation. Firstly, we demonstrated that a significantly higher proportion of CALR patients displayed EMC in comparison to “triple negative” and to MPL-mutated patients. CALR mutated patients also expressed a higher number of EMC compared to JAK2V617F-mutated patients. More frequent EMC and higher number of EMC in CALR mutated patients argue for an impact of CALR on autonomous MK proliferation. In the same way, it is consistent with the quasi absence of CALR mutations in Polycythemia Vera (13) despite rare PV cases with CALR mutations have recently been reported (14), the higher platelet counts in CALR patients (11) and the preferential expression of CALR mutated protein in megakaryocytes (15). Our results also confirm that EEC is characteristic feature of JAK2V617F mutation. No EEC was observed in MPL mutated patients and only one patient carrying CALR mutation was EEC positive. These functional differences could be related to lower hemoglobin counts observed in CALR patients compared to JAK2V617F ones. These differences in the balance between erythroid and MK proliferation strengthen the evidence for a megakaryocytic role for CALR mutants in ET pathogenesis and define different growth
profiles of ET: JAK2V617F patients harbor a “mixed profile” (erythroid and MK) compared to the “MK profile” of CALR patients.

Among populations presenting EMC, we observed significantly more EMC and a higher allelic burden in type 2 than in type 1. This supports the hypothesis that in CALR mutated patients, the allele burden increase induces increased cytokine-independent MK proliferation and subsequent clone enlargement. This suggests not only a contribution of mutation type but also of CALR allele burden on ET phenotypes. Nevertheless, the CALR allelic burden did not show any linear correlation, either in type 1 or in type 2 mutants, with the number of EMC. Hence, the CALR allelic burden may not be the only factor determining the intensity of EMC growth: others mutations or genetic modifiers (i.e. epigenetic, RNA splicing) could impact MK activation and thereby disease evolution.

Finally, our work showed that “variant” subpopulation displayed EMC in 50% of cases. Even though MK “variants” appear less proliferative (less frequent EMC) than type 1 CALR, a linear and positive correlation between the number of EMC and allelic burden was observed. This suggests that in this heterogeneous subgroup, EMC could identify patients in whom CALR plays a pivotal role in autonomous activation of MK. In our opinion, it may be interesting to study “not type 1 nor type 2” variants in a larger cohort and according to their clinical features.

In conclusion, our study underlines the functional link between CALR mutants and megakaryocyte growth stimulation and provides findings that improve our understanding of phenotypic differences observed among patients with different drivers mutation and different CALR genotypes.

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References:


Figure legends

Figure 1: Proportion of patients presenting respectively EMC growth according to mutational status in ET. Statistical differences between mutations were analyzed in pairs (Chi² test). * means p value <0.05; ** means p value <0.01.

Figure 2: Distribution of number of Endogenous Megakaryocytic Colonies (EMC) among CALR population showing EMC growth. “Variant” corresponds to neither type 1 nor type 2 mutations. Whisker plots represent numbers of EMC expressed per 10⁵ cells plated according to type of CALR mutation. * means p value<0.05.

Figure 3: Linear regression of CALR allelic burden according to number of endogenous megakaryotic colonies (EMC). Each symbol represents a patient (respectively + for type 1, ▲ for type 2, ■ for “variants”). Linear regression was done according to type of CALR mutations (−− for type 1, ----for type 2 and — for “variants”).