Absence of *CALR* mutations in *JAK2*-negative polycythemia

Polycythemia vera (PV) is a myeloproliferative neoplasm (MPN) responsible for increased hematopoiesis, mainly affecting the erythroid lineage. The diagnosis of PV has been simplified enormously by the description of *JAK2* mutations, affecting the canonical V617 in exon 14 in more than 95% of cases, or the exon 12 in the majority of JAK2V617F negative patients. In addition to finding a *JAK2* mutation in patients with increased hemoglobin/hematocrit levels, the World Health Organization (WHO) in its 2008 classification put forward additional "minor" criteria, such as low serum erythropoietin (EPO) levels, a growth of endogenous (EPO-independent) erythroid colonies, and specific bone marrow histological features of MPN.[1] In the most recent classification,[2] bone marrow biopsy has become a major criterion, whereas only subnormal EPO levels remain a minor criterion. Even though *JAK2* mutations are found in the vast majority of PV patients, "true" PV has been described in patients lacking mutations in the exon 12 or 14 of *JAK2*, raising the question of other mutations causing this phenotype. For example, among these possible mutations, abnormalities in the adaptor protein LNK have been reported in *JAK2*-unmutated patients.[3]

In 2013, a novel series of mutations affecting the *Calreticulin (CALR)* gene have been described in *JAK2*-unmutated essential thrombocythemia and primary myelofibrosis.[4,5] *CALR* mutations are found in the exon 9 and consist of various combinations of deletions and insertions that always result in a one base-pair frameshift. This constant frameshift changes the C-terminus of the protein that becomes basic, whereas it is very acidic in the wild-type protein. Even though no *CALR* mutations had been initially described in PV, the fact that this mutation activated the JAK2/STAT5 pathway led some authors to investigate whether CALR mutations can be found in PV patients lacking *JAK2* mutations. Two patients with erythrocytosis harboring *CALR* mutations have been described,[6] which calls into question the interest of systematically screening for *CALR* mutations in patients with unexplained erythrocytosis.

First, we screened a cohort of 42 patients gathered nationally on the basis of a PV diagnosis, but without *JAK2* mutations, either in exon 12 or 14 ("JAK2NEG cohort"). All patients gave informed consent for the use of remaining nucleic acids for research purposes after the completion of diagnostic procedures. *CALR* exon 9 mutations were screened by fragment length analysis according to procedures described by Klampfl *et al*.[5] and/or to Mansier *et al*.[7]

Of these 42 PV patients, a *CALR* mutation (type 1, c.1092_1143del; p.Leu637Trpfs*46) was found in one case. The patient was a 71-year old woman with mild thrombocytosis (573 x10^9/L), for whom a systematic isotopic evaluation had revealed an increased red cell mass (130%). The white blood cell count was normal (8.5x10^9/L) and she had no splenomegaly. The diagnostic workup included search for mutations in *JAK2* (exon 12 and 14), MPL, BCR-ABL1 and a karyotype; all were normal. The bone marrow biopsy was suggestive of MPN, showing mainly hyperplasia of the megakaryocytic lineage. An *in vitro* assay of progenitors revealed an EPO-independent growth of small erythroid colonies. This suggested a diagnosis of PV, even though the hemoglobin level was within the normal range (13.5 g/dL). In view of the surprising finding of a *CALR* mutation, clinical and biological diagnosis criteria have been critically reviewed. A central re-evaluation of the bone marrow histology confirmed the isolated megakaryocytic hyperplasia that was more in favor of a diagnosis of ET than PV. Moreover, chronic hypoxia (pO2 67 mmHg, oxygen saturation of 94%) had been overlooked. In light of these novel elements, the patient was re-classified as having essential thrombocythemia associated with a secondary erythrocytosis due to subnormal oxygen saturation.

To strengthen this study, the *CALR* mutation screening was then extended to another cohort of 536 patients diagnosed with erythrocytosis from three separate laboratories. Only cases where no typical causes of secondary erythrocytosis and no *JAK2* mutations (exon 12, exon 14) had been identified were studied. From this large cohort, only one additional *CALR* mutation was found. The 67-year old patient had hematocrit levels of 53%-56%, showed only a modest increase in red cell mass (135%), no clinical sign of PV, no endogenous erythroid colony growth, and a serum EPO level of 10 mIU/mL. The patient's body mass index was 34.7. The bone marrow biopsy only revealed increased erythroid lineage, without alteration of the other lineages. A thorough search for the causes of secondary erythrocytosis (abdominal ultrasound, blood gas, P50 measurement, methaemoglobinemia, hemoglobin electrophoresis, overnight sleep monitoring) remained negative. A type 1 *CALR* mutation with low allele burden (estimated at 5%) was found in peripheral leukocyte DNA as well as 2 out of 12 EPO-stimulated erythroid colonies.

Overall, of 578 patients with JAK2 negative unexplained erythrocytosis, 2 presented a *CALR* exon 9 mutation: one with evidence of essential thrombocythemia and slightly increased red cell mass (without increased hemoglobin) probably due to suboptimal hemoglobin oxygen saturation, and one with an idiopathic erythrocytosis, no elements in favor of an MPN (no endogenous erythroid colony growth, normal EPO levels, no sign of myeloproliferation on the bone marrow histology) and a low CALR mutant burden. In this last case, it is difficult to attribute the responsibility of the increased hemoglobin to the *CALR* mutation. Rather one may postulate that it is a chance association of erythrocytosis in an obese man and the presence of an asymptomatic, randomly acquired mutation has been described to occur quite frequently with age.[8] Of note, *CALR* mutant allelic burden increased over time up to 20%, without changing the clinical presentation.

Two groups had previously reported 3 cases of polycythemic patients with *CALR* mutations. Xu *et al*. described a 3bp deletion of *CALR* (c.1095_1097del), which is probably a polymorphism.[9] Indeed, this mutation does not generate the typical frameshift observed in *CALR* mutations, and several such cases have been identified as non-pathogenic genetic variants.[10] Regarding the 2 other published cases,[6] WHO criteria for PV were not complete. In the absence of *JAK2* mutations, two of the three minor criteria are required for a diagnosis of PV according to the 2008 classification, or a biopsy and a low EPO level according to the 2016 classification (absence of low erythropoietin levels, no endogenous erythroid colony investigation for the first patient, absence of endogenous erythroid colony formation and no bone marrow biopsy investigation for the second one). It is, therefore, possible that these patients did not suffer from a "true" PV. Furthermore, CALR mutants have recently been shown to interact specifically with MPL, explaining the major involvement of these mutations in ET and PMF, which strongly rely on abnormal MPL signaling.

Moreover, the introduction of CALR mutants in murine hematopoietic cells induces thrombocytosis without erythrocytosis.[11-14] For these reasons, and in view of our results indicating that *CALR* mutations were not found in a large cohort of patients with unexplained erythrocytosis, we argue that screening for *CALR* mutations in polycythemic patients is not useful.

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