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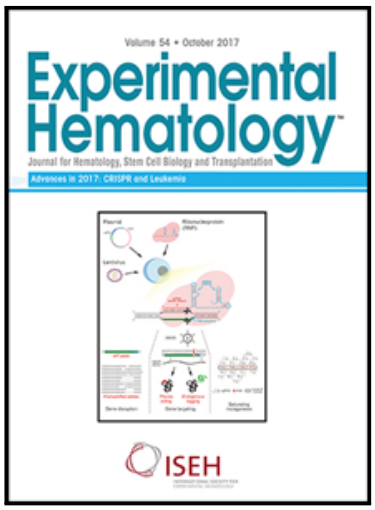

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Skewed ratio between type 1 and type 2 CALR mutations in Essential Thrombocytosis patients with concomitant JAK2 V617F mutation

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Highlights

- Dominant CALR clone in Essential Thrombocytosis with low frequency JAK2 V617F.
- CALR clone dictates the disease phenotype in ET with concomitant JAK2 V617F.
- Extended molecular diagnostics is warranted in low frequency JAK2 V617F MPNs.

Abstract

Detection of somatic mutations in cardinal driver genes is a strong argument for diagnosis in classical Philadelphia-negative myeloproliferative neoplasms (MPN). Driver mutations in JAK2, CALR and MPL, are generally considered mutually exclusive, but several reports have suggested that they co-exist in a small subgroup of patients.

In this study we retrospectively analyzed for CALR mutations in 136 suspected MPN patients with low allelic burden (≤5%) JAK2 V617F. Fifteen patients with concomitant JAK2 V617F and CALR mutation were identified of which ten were diagnosed with essential thrombocytosis (ET). More than 50 different indel mutations in exon 9 of CALR have been reported, with type 1 (52 bp deletion) and type 2 (5 bp insertion) accounting for more than 80% of CALR mutated MPN cases. Type 1 is generally considered the most common mutation, but interestingly, our double mutated ET patients seem to have an inversed ratio between type 1 and type 2 CALR mutations.

Our findings support the possibility of co-existing JAK2 V617F and CALR mutations and stress the importance of further molecular screening in MPN patients with low allele frequencies of JAK2 V617F.

Keywords

Essential thrombocytosis; CALR; JAK2 V617F; myeloproliferative neoplasms; driver mutations

Introduction

The classical Philadelphia-negative myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF), are a group of clonal hematopoietic stem cell disorders characterized by the overproduction of terminally differentiated and fully functional hematopoietic cells. Major molecular diagnostic criteria for the MPNs, include presence of somatic mutations in cardinal driver genes; JAK2 V617F or JAK2 exon 12 in PV and JAK2 V617F, CALR exon 9 or MPL exon 10 in ET and PMF[1].

The MPN driver mutations are generally considered mutually exclusive and with detection of the common JAK2 V617F mutation usually no further testing is performed. However, in recent years papers have been published suggesting that driver mutations indeed do co-exist[2-4].

Detection of such 'double mutant' patients could very well have clinical implications. There is a general consensus that the mutation type affects the phenotypic manifestations and prognosis. Patients with JAK2 mutation often exhibit more prominent leukocytosis, lower levels of platelets, higher hemoglobin levels, more thrombotic events and chance of transformation into AML. CALR mutations are characterized by lower risk of thrombosis, lower hemoglobin and leukocyte counts, higher platelets levels and a better overall survival [5, 6]. We wanted to address the issue of double mutants in our patients to ensure adequate molecular characterization for aiding clinicians providing more precise diagnoses and prognostication in patients with PMF and ET.

Methods

In this study, we retrospectively reanalysed for CALR mutation in all suspected MPN patients initially analysed for JAK2 V617F between January 2012 and April 30th 2015 for whom allele frequencies of the JAK2 mutation was between 0.01% and 5%. 136 patients were positive for JAK2 V617F with allele frequencies in this range. In addition, a smaller test group of 46 patients with JAK2 V617F allele frequencies between 6% and 10% were analysed. The JAK2 V617F analysis was performed with allele specific qPCR with sensitivity at 0.01% as described previously [7]. CALR exon 9 mutations were analysed by fragment length analysis with sensitivity at 2% as described previously [8]. All samples positive for a CALR mutation were confirmed by Sanger sequencing to determine the specific type of CALR exon 9 mutation. Statistical analyses were performed by Fisher's Exact Test and Student's T-test. The study was approved by the local ethical committee and the Danish Data Protection Agency.

Results and discussion

We identified 15 patients with concomitant CALR mutation within the group of 136 patients with JAK2 V617F allele frequencies (VAF) ≤5% while none of the 46 patients with JAK2 V617F VAF >5% had a CALR mutation (p=0.013). Clinical data, collected at diagnosis prior to any treatment, and mutational status of the 15 double mutated patients can be seen in Table 1. The identification of concurrent CALR mutation within the group of suspected MPN patients with low JAK2 V617F allele frequencies is in line with previous studies. Mansier *et al* reported concurrent CALR mutation in only 2 of 412 patients (0.5%) with JAK2 V617F VAF >5% while in 19 of 133 patients (14%) with JAK2 V617F VAF <5% and Usseglio *et al* observed concurrent JAK2 V617F and CALR/MPL mutation exclusively in patients with JAK2 V617F VAF<5% [2, 3].
Ten of our double mutated patients were diagnosed with ET, three with PMF and two patients were MPN-unclassifiable. JAK2 V617F and CALR double mutation have been previously observed in both ET and PMF but with at preponderance for ET as also observed in the current study [3, 9, 10].
More than 50 different indel mutations in exon 9 of CALR have been found with type 1 (52 bp deletion) and type 2 (5 bp insertion) as the most common variants. In our double mutated patients we identified five type 1 (33%) and ten type 2 (66%) CALR mutations and in the ET group 3 patients were found positive for CALR mutation type 1 (30%) and seven were positive for type 2 (70%). More type 1 (53%) than type 2 (32%) CALR mutations are found in MPN patients in general [8] while the rest are classified as either type 1-like, type2-like or 'other types', based on the remaining stretches of negatively charged amino acids in the mutant proteins [11]. The distribution of CALR mutations is hence 57% type 1-like and 39% type 2-like in ET, and 83% type 1-like and 15% type 2-like in PMF [11]. The clinical significance of type 1 versus type 2 CALR mutations is, however, debated. A recent report indicate that type 1 mutations are associated with PMF or ET with an increased risk of myelofibrotic transformation, while type 2 mutations are associated with ET, low risk of thrombosis and indolent clinical course[11]. Another report on ET patients showed that patients with type 2 variants displayed significantly higher platelet count but that the two CALR mutation variants were similar in their hemoglobin level, leukocyte count, IPSET (International Prognostic Scoring System for Essential Thrombocythemia) scores and in overall and thrombosis-free survival [12]. A third report suggests that the prognostic advantage of CALR mutations in PMF might be confined to type 1 or type 1-like CALR variants [13].
The skewed frequencies of type 1 versus type 2 mutations observed in the present study could, however, indicate that double mutated patients constitute a distinct MPN entity. This hypothesis is supported by a recent report that found an association between concurrent JAK2 V617F and CALR/MPL mutation and higher age compared to single mutated ET patients [9]. However, this could not be confirmed in our cohort where the mean age of our double mutated ET patients was 64.1 years while 59.3 years in 66 ET patients diagnosed at our institution with CALR mutation alone (p=0.31).
Our double mutated ET patients had median thrombocyte count at 1195 $x10^6$/l. Erythrocyte volume fraction was within normal range for all ET patients (not shown), one patient had slightly lowered hemoglobin level, and all except one

patient had moderate increased lactate dehydrogenase (Table 1). Two of 3 ET patients with type 1 mutation experienced deep venous thrombosis (DVT) or Non ST-Elevation Myocardial Infarction (non-STEMI) two years prior to diagnosis while only one patient out of seven with type 2 mutation had DVT two years post diagnosis. Even though this is a small study, these results could imply that type 1 CALR mutations are more frequently associated with thrombosis than type 2 CALR mutations as also suggested previously [11]. None of the ET patients progressed to myelofibrosis during a median follow-up time at 59 months and all ET patients were alive at end of follow-up.

We searched for CALR mutations in 136 patients with low allele burden JAK2 V617F (≤5%) and identified CALR mutations with allele frequencies in the range 9 - 44% in 15 (11%) of these patients, stressing that the CALR clone is the dominant one in our double mutated cases in accordance with previous studies [2, 3]. This finding, in combination with the high thrombocyte count and few thrombotic events in ET patients with type 2 mutation, indicate that the CALR clone and mutation subtype dictate the disease phenotype in double mutant patients. Future studies are warranted for clarifying the etiology of double mutated MPN patients and whether mutations arise in the same or two different tumor clones.

In conclusion, our data stress the importance of extending the molecular screening in suspected MPN patients with low allele frequencies of JAK2 V617F (*e.g* ≤3%) to also include CALR in order to confirm the MPN diagnosis and refine prognostication.

Conflict of interest disclosure:

The authors declare no competing financial interests.

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Table 1. Clinical and mutational characteristics of the fifteen patients with concomitant JAK2 V617F and CALR mutation. VAF: variant allele frequency, LDH: lactate dehydrogenase, DVT: deep venous thrombosis, non-STEMI: non ST-Elevation Myocardial Infarction, NA: not available. Numbers in bold font indicate levels outside normal range.

Patient ID	Sex	Diagnosis	Age at diagnosis	JAK2V617F (VAF%)	CALR mutation	CALR (VAF %)	Hemoglobin (mmol/L)	Platelets (x10^9/L)	Leukocytes (x10^9/L)	LDH (U/L)	Thrombosis	Follow-up (months)
1	M	ET	75	0.02	Type 1	44	8.8	**1076**	**11**	**321**	non-STEMI	69
2	F	ET	58	0.02	Type 1	28	9.2	**1273**	6	**289**	DVT	62
3	F	Unclassifiable	85	0.01	Type 2	27	8.5	**859**	9.1	**335**		33
4	F	PMF	49	0.01	Type 1	15	9.3	229	6.9	**289**	DVT	43
5	F	ET	69	0.06	Type 2	35	9.4	**1010**	8.6	**228**		52
6	F	ET	54	0.09	Type 2	33	7.7	**1354**	7.3	**219**		56
7	M	PMF	67	0.1	Type 1	40	**7.4**	**674**	**11.1**	**491**		48
8	F	ET	72	0.5	Type 2	41	8.2	**1584**	**12.7**	**292**		55
9	M	ET	47	0.01	Type 2	43	9.2	**1881**	10	**378**		204
10	M	ET	61	0.02	Type 2	44	9.5	**906**	6.5	NA		154
11	M	ET	77	0.03	Type 2	36	**8.0**	**1131**	9.7	**412**	DVT	40

12	M	ET	52	0.1	Type 2	41	9.7	**1259**	9	190	145
13	F	PMF (5q-)	74	1	Type 2	25	**5.8**	73	8.5	**570**	46
14	M	Unclassifiable	57	3	Type 2	9	9.8	**617**	7.8	182	43
15	M	ET	76	3	Type 1	32	8.6	**715**	**10.2**	**274**	49

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