MYELOID NEOPLASIA

CALR exon 9 mutations are somatically acquired events in familial cases of essential thrombocythemia or primary myelofibrosis

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Key Points

- Somatic indels of *CALR* exon 9 are present in about 20% to 25% of sporadic patients with essential thrombocythemia or primary myelofibrosis.
- These mutations are found also in familial cases of essential thrombocythemia or primary myelofibrosis as somatically acquired events.

Somatic mutations in the calreticulin (*CALR*) gene were recently discovered in patients with sporadic essential thrombocythemia (ET) and primary myelofibrosis (PMF) lacking *JAK2* and *MPL* mutations. We studied *CALR* mutation status in familial cases of myeloproliferative neoplasm. In a cohort of 127 patients, *CALR* indels were identified in 6 of 55 (11%) subjects with ET and in 6 of 20 (30%) with PMF, whereas 52 cases of polycythemia vera had nonmutated *CALR*. All *CALR* mutations were somatic, found in granulocytes but not in T lymphocytes. Patients with *CALR*-mutated ET showed a higher platelet count ($P = .017$) and a lower cumulative incidence of thrombosis ($P = .036$) and of disease progression ($P = .047$) compared with those with *JAK2* (V617F). In conclusion, a significant proportion of familial ET and PMF nonmutated for *JAK2* carry a somatic mutation of *CALR*. (*Blood*. 2014;123(15):2416-2419)

Introduction

Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), have in most instances sporadic occurrence.[1] However, familial clustering of MPN has been reported by us and others.[2,3] Common mutations involved in the pathogenesis of MPN such as *JAK2*, *MPL*, *CBL*, and *TET2* are mainly somatically acquired also in familial cases,[3-5] although rare cases of germline *MPL*, *CBL*, and *TET2* mutations have been reported.[6-8] The *JAK2* GGCC haplotype confers susceptibility to MPN, but does not explain familial clustering.[5]

Two recent articles demonstrated that most of the patients with sporadic ET or PMF not associated with *JAK2* or *MPL* alterations carry somatic mutations of calreticulin (*CALR*).[9,10] The clinical course of sporadic *CALR*-mutated patients is more indolent than that of *JAK2*-mutated patients.[9]

The role of *CALR* mutations in familial MPN remains to be clarified. In this study, we aimed to investigate the frequency of *CALR* mutations in familial MPN, their germline or somatic occurrence, and their correlation with clinical phenotype.

Patients and methods

This study was approved by the Ethics Committee of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy. The procedures followed were in accordance with the Helsinki Declaration, and samples were obtained with patients' written informed consent.

We identified in our database a total of 154 consecutive patients with familial MPN diagnosed and followed from 1970 to 2013 at the Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia. DNA was available in 127 cases belonging to 78 families, which were included into the study. Patients were defined as familial cases if 2 or more individuals within the same pedigree were affected.[2] Diagnosis of MPN was made in accordance with the criteria in use at the time of the first observation, as previously described.[11]

JAK2 (V617F) mutation status was assessed in granulocyte DNA as previously described.[12] *JAK2* wild-type patients were further evaluated for *JAK2* exon 12 mutations, *MPL* exon 10 mutations, and *CALR* exon 9 mutations.[9,13-16] Patients with nonmutated *JAK2*, *MPL* exon 10, and *CALR* exon 9 were sequenced for the other 8 exons of *CALR*.[9] Female patients belonging to pedigrees with all affected members negative for *JAK2*, *MPL*, and *CALR* were evaluated for clonality using X-chromosome–inactivation pattern (XCIP).[12]

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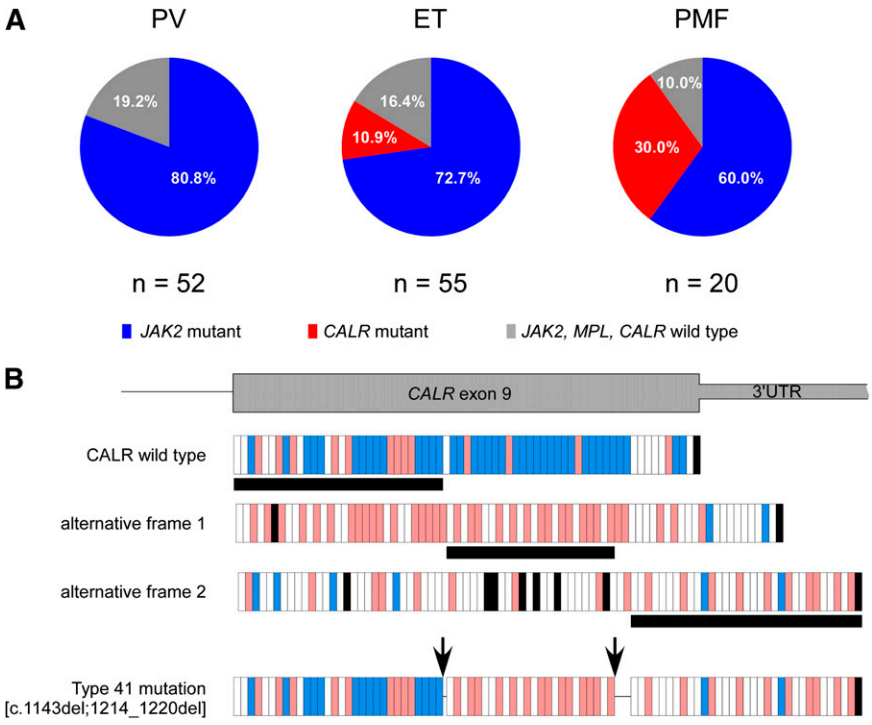

Figure 1. Distribution of mutations according to diagnosis and description of the novel *CALR* mutation. (A) *JAK2* mutations were present in 42 of 52 (80.8%) patients with PV, 40 of 55 (72.7%) patients with ET, and 12 of 20 (60%) patients with PMF. *CALR* mutations were identified in 6 of 55 (10.9%) patients with ET and 6 of 20 (30%) patients with PMF. The remaining 10 patients (19.2%) with PV, 9 patients (16.4%) with ET, and 2 patients (10%) with PMF did not carry any mutation. (B) The novel type 41 is a complex mutation, consisting of 2 separate deletion events of 1 bp and 7 bp. These 2 deletions were shown to be on the same allele by polymerase chain reaction product subcloning and sequencing, as described previously.[9] The first deletion introduces a frameshift to alternative frame, which is common to all *CALR* exon 9 mutations, whereas the second deletion causes a frameshift to the third alternative frame at the end of exon 9. Because the next stop codon comes later in the third frame, this mutation creates the longest CALR protein described so far (20 amino acids longer than the wild type). Each vertical bar represents an amino acid: blue bars indicate negatively charged amino acids, red bars indicate positively charged amino acids, white bars indicate uncharged amino acids, and black bars indicate stop codons. Horizontal black bars below the frames denote the amino acid stretch encoded by the respective frame. The arrows indicate the location of the 2 deletions. 3′ UTR, 3′ untranslated region of the gene.

Overall survival was estimated using the Kaplan-Meier product limit method, and survival curves were compared by the log-rank test. The cumulative incidence of disease transformation and that of thrombotic events were estimated with a competing risk approach, considering death from all causes as a competing event.[17] The comparison of cumulative incidence curves in different groups of patients was carried out using the Pepe-Mori test.[18] All *P* values were considered statistically significant when smaller than .05 (2-tailed). Statistical analyses were performed using Stata12.1 (StataCorp LP, College Station, TX) software.

Results and discussion

Of 127 patients, 94 carried *JAK2* mutations (91 *JAK2* V617F and 3 exon 12 mutations), 12 carried *CALR* mutations, and 21 did not carry any mutations. We did not find any *MPL* mutation. The distribution of mutations according to diagnosis was significantly different ($P = .003$), with *CALR* mutations being associated only with ET and PMF, as reported in Figure 1A.

All *CALR* mutations were demonstrated to be somatically acquired by genotyping T-cell DNA and showing the absence of *CALR* mutations in that control tissue. The *CALR* exon 9 mutations found in familial MPN patients are listed in Table 1. As reported previously for sporadic MPN,[9] the type 1 (n = 4) and type 2 (n = 6) *CALR* mutations were predominant, with the remaining 2 patients carrying type 27 and a novel type (which we numbered continuously as type 41) *CALR* mutation. The novel type 41 is a complex mutation, consisting of 2 separate deletion events of 1 bp and 7 bp, as reported in Figure 1B; this mutation creates the longest mutant CALR protein described so far.

The clinical phenotype within the familial cluster was homogenous in 41 (53%) of 78 families (PV in 20 families, ET in 16 families, PMF in 5 families), whereas the remaining 37 families (47%) exhibited mixed phenotypes. In 45 of 78 families, DNA was available for all affected members; the somatic mutational and diagnosis pattern are reported in supplemental Table 1 on the *Blood* Web site. One family showed a homogeneous phenotype (ET) and coexistence of *JAK2* and *CALR* mutations (supplemental Figure 1).

Table 1. Somatic *CALR* mutations found in familial MPN patients

Patient ID	Sample ID	Diagnosis	*CALR* mutation type	*CALR* mutation	Mutation burden, %
S47A9SM	MPC09_213	PMF	1	c.1092_1143del	27
S48A9SM	MPC09_214	PMF	1	c.1092_1143del	40
S12A12SM	MPC12_22	PMF	1	c.1092_1143del	52
S29A12SM	MPC12_115	ET	1	c.1092_1143del	48
S48A7SM	MPC07_162	ET	2	c.1154_1155insTTGTC	24
S106A7SM	MPC07_395	PMF	2	c.1154_1155insTTGTC	44
S20A10SM	MPC10_100	ET	2	c.1154_1155insTTGTC	27
S113A10SM	MPC10_288	ET	2	c.1154_1155insTTGTC	40
S157A10SM	MPC10_594	PMF	2	c.1154_1155insTTGTC	47
S100A11SM	MPC11_627	ET	2	c.1154_1155insTTGTC	47
S167A6SM	906	ET	27	c.1123_1125delinsTGTTT	50
S17A99SM	f5p3	PMF	41	[c.1143del;1214_1220del]	49

Interestingly, there were 3 families with all patients diagnosed with PV and nonmutated *JAK2* and 3 families with all patients diagnosed with ET and nonmutated *JAK2, MPL,* and *CALR* (supplemental Table 2). Twelve of 14 samples from these 6 families were analyzed by Affymetrix SNP 6.0 arrays: 10 had normal karyotype and 2 had aberrations of the Y chromosome, which can be also related to aging. Five female patients were evaluated for XCIP: 2 samples were polyclonal, 2 were not evaluable, and 1 had ambiguous XCIP. Affected members of 1 of our families diagnosed with ET were recently found to carry a germline *JAK2* mutation,[16] as also shown by other investigators.[19,20] Overall, absence of somatic mutations, normal karyotype, and polyclonal hematopoiesis in cases of familial ET or PV with nonmutated *JAK2, MPL,* and *CALR* suggest that these patients are very likely to be cases of hereditary thrombocytosis or erythrocytosis.[21,22]

We did not find significant differences in terms of clinical phenotype (age, sex, leukocyte count, hemoglobin, platelet count, erythropoietin, splenomegaly, thrombosis) at diagnosis according to genotype in ET and PMF patients, perhaps because of the low number of patients (supplemental Tables 3 and 4). The only significant difference ($P = .017$) was a higher platelet count in *CALR*-mutated ET patients (median 1222×10^9/L, range 563-1536) in comparison with *JAK2*-mutated ET patients (median 657×10^9/L, range 456-1500), confirming what was previously observed in ET sporadic cases.[9,11,23]

The whole cohort was observed for a median follow-up from diagnosis of 8.4 years (range, 0-32.8 years). Of 127 patients, 20 died; 17 (13.4%) patients had thrombotic events and 11 (8.7%) patients developed disease progression (7 secondary myelofibrosis and 4 leukemic evolutions). Cumulative incidences of thrombosis and disease progression at 10 years according to diagnosis and mutational status are reported in supplemental Table 5.

The molecular status (*CALR*-mutated vs *JAK2*-mutated vs nonmutated *CALR/JAK2/MPL*) did not affect overall survival in patients with ET and PV, whereas it did in patients with PMF ($P = .001$). In detail, in PMF, the median overall survival was 1.3 years in patients with nonmutated *CALR/JAK2/MPL* and not reached in *CALR*-mutated and *JAK2*-mutated patients, suggesting a worse prognosis in *CALR/JAK2/MPL*-unmutated PMF, as recently observed in sporadic cases.[24] In ET, *CALR*-mutated patients showed a lower incidence of thrombosis (10-year confidence interval 0% vs 12.8%, $P = .036$) and a lower incidence of disease progression (10-year confidence interval 0% vs 6.6%, $P = .047$) in comparison with *JAK2*-mutated patients (supplemental Figure 2A-B), although the low number of patients does not allow any firm conclusion. The lower rate of thrombosis in *CALR*-mutated ET is consistent with what we previously observed in sporadic ET.[9,11]

In conclusion, in familial MPN, *CALR* mutations are somatically acquired and are associated with ET or PMF phenotype, as in sporadic MPN. In addition, in familial ET, *CALR* mutations are associated with a lower risk of thrombosis with respect to *JAK2* (V617F), as observed in sporadic ET.

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Authorship

Contribution: E.R., A.S.H., R.K., and M.C. conceived this study, collected and analyzed data, and wrote the manuscript; I.C.C., M.B., C.C., E.S., and C.A. collected clinical data; A.S.H., D.P., J.D.M., N.C.C.T., C.M., T.B., and R.K. did molecular investigations; V.V.F. and C.P. did statistical analyses; and E.B. studied bone marrow biopsies.

Conflict-of-interest disclosure: R.K. reports having a pending patent application on the use of calreticulin gene mutations for the diagnosis and therapeutic targeting of MPNs. The remaining authors declare no competing financial interests.

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