Brief Report

MYELOID NEOPLASIA

# X chromosome inactivation analysis reveals a difference in the biology of ET patients with *JAK2* and *CALR* mutations

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## Key Points

- In ET, a *CALR* mutation correlates with a monoclonal X chromosome inactivation pattern, which differs from *JAK2*$^{V617F}$ mutant disease.
- The presence of a *CALR* mutant is associated with suppression of wild-type myelopoiesis.

**Calreticulin mutations (*CALR*$^{MUT}$) are found in a significant proportion of patients with essential thrombocythemia (ET) lacking *JAK2*$^{V617F}$ or *MPL* mutations. They are associated with substantially different hematological and clinical features and define a distinct subtype of ET. We show here that their presence is significantly correlated with a clonal X chromosome inactivation pattern (XCIP). Of 105 female ET patients investigated, 61 had an interpretable XCIP, and a clonal pattern was observed in 88% of *CALR*$^{MUT}$ patients compared with 26% of *JAK2*$^{V617F}$ ($P = .0002$) and 9% of *JAK2*$^{V617F}$/*MPL*/*CALR* wild-type patients ($P < .0001$). Neutrophil *CALR*$^{MUT}$ level was significantly higher than *JAK2*$^{V617F}$ level (median, 50% vs 18%; $P < .0001$), and wild-type myelopoiesis was suppressed in *CALR*$^{MUT}$ but not *JAK2*$^{V617F}$ patients. These data are suggestive of truly monoclonal hematopoiesis in *CALR*$^{MUT}$ patients and provide further evidence that the biology associated with *CALR* mutations is markedly different from that of *JAK2*$^{V617F}$ mutations. (*Blood*. 2014;124(13):2091-2093)**

## Introduction

Discriminating between a reactive thrombocytosis and the myeloproliferative neoplasm essential thrombocythemia (ET) can be a diagnostic challenge. In the absence of known clonal biomarkers, several groups, including our own, investigated clonality in female patients using X chromosome inactivation patterns (XCIPs) to distinguish between them on the premise that ET was a clonal disorder.[1-4] However, these studies demonstrated considerable biological heterogeneity in ET patients, with 3 broad XCIP categories observed once constitutional and age-related skewing had been taken into account: monoclonal, oligoclonal, or polyclonal. Identification of *JAK2*$^{V617F}$ mutations provided the first molecular biomarker in ET, but this also led to a biological conundrum as their presence did not appear to relate to clonality status, with all 3 patterns observed in mutant-positive patients.[5-8] Subsequent studies reported that the relative *JAK2*$^{V617F}$ mutant allele burden is generally lower than would be expected for a mutation driving clonal proliferation,[8-13] and therefore in many mutant-positive patients, a significant proportion of the platelets are mutant negative and polyclonal in origin. Serial analysis of neutrophil samples has shown that *JAK2*$^{V617F}$-mutated clones can be maintained as a stable subpopulation for many years, even in the absence of treatment.[10-13] Furthermore, we demonstrated that in *JAK2*$^{V617F}$ cases, the observed neutrophil XCIP was very similar to that expected when the mutant level and constitutional (T-cell) XCIP were taken into account.[10] Of note, in all these studies, a significant proportion of ET patients with clonal XCIPs were *JAK2* wild type, implying the presence of other mutations leading to clonal expansion. *MPL* exon 10 mutations were later detected, but only account for 3% to 9% of ET patients lacking *JAK2*$^{V617F}$ mutations.[14,15]

Recently, calreticulin mutations (*CALR*$^{MUT}$) have been reported in 49% to 71% of ET patients lacking *JAK2*$^{V617F}$ or *MPL* mutations that define a distinct subtype of ET with a substantially different hematological phenotype and clinical outcome from *JAK2*$^{V617F}$-mutated patients.[16-19] However, clonality status in these patients has not been reported. We therefore screened a cohort of 105 female patients with ET and known *JAK2* and *MPL* genotype for *CALR* mutations and correlated the results with XCIPs in the 61 with interpretable patterns.

## Study design

The median age at the first test for the 105 female patients investigated was 50 years (range, 10-92 years). Peripheral blood samples were collected between 1996 and 2010. The diagnosis was made according to the criteria for ET at the time of the first investigation (amended Polycythemia Vera Study Group or revised World Health Organization criteria). The studies were approved by the London Multi-centre Research Ethics Committee. Patient consent was obtained according to the Declaration of Helsinki.

XCIPs of purified neutrophil and CD3$^+$ T-cell DNA were investigated using the human androgen receptor assay.[10] As established in our previous studies, a clonal XCIP required skewed neutrophil X allele expression of >75% of one X allele, >20% difference between X allele expression in neutrophils and T cells (reflecting the hematopoietic stem cell pattern), and age ≤ 65 years at the time of test.[20] Neutrophil *JAK2*$^{V617F}$ genotype and mutant allele level were determined as previously reported.[10] Neutrophil DNA was screened for mutations in *MPL* exon 10 (primers 5′-GTGGGCCGAAGTCT GACCCT-3′, 5′-CGCTCTGTGACCCCAGATCTC-3′) and *CALR* exon 9 (primers 5′-GCCTGGTCCTGGTCCTGATGT-3′, 5′-AGGAGCGCTCAGG

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**Table 1. Characteristics of the *CALR*$^{MUT}$ patients studied compared with *JAK2*$^{V617F}$ and triple-negative patients**

| Parameter | Total | *JAK2*$^{V617F}$ | *CALR*$^{MUT}$ | Triple negative | *P* value for *CALR*$^{MUT}$ vs *JAK2*$^{V617F}$ | *P* value for *CALR*$^{MUT}$ vs triple negative |
|---|---|---|---|---|---|---|
| No. (% of cohort) | 105* | 43 (41%) | 26 (25%) | 34 (33%) | | |
| Age at diagnosis, years, median (range)† | 47 (10-84) | 54 (25-84) | 31 (10-71) | 40 (14-77) | .0002 | .2 |
| Platelet count at diagnosis, $\times 10^9$/L, median (range)‡ | 807 (456-4875) | 800 (523-1140) | 1169 (500-4875) | 721 (456-2800) | .001 | .1 |
| Age at first test, years, median (range) | 50 (10-92) | 61 (25-92) | 37 (10-89) | 49 (16-89) | .003 | .8 |
| Months from diagnosis at first test, median (range)† | 18 (0-297) | 20 (0-217) | 40 (0-175) | 12 (0-297) | .5 | .3 |
| % Mutant, median (range) | | 18% (5-100%)§ | 50% (22-56%) | | <.0001 | |
| % Mutant at or within 3 mo of diagnosis, median (range) | | 17% (10-42%) | 46% (27-56%) | | .0001 | |
| XCIP status, clonal:polyclonal (%) | 22:39 (36%:64%) | 6:17 (26%:74%) | 14:2 (88%:12%) | 2:20 (9%:91%) | .0002¶ | <.0001¶ |

*P* values are the Student unpaired *t* test unless otherwise stated.
*Two patients were *MPL*$^{MUT}$.
†Date of diagnosis was unknown in 10 patients: 2 *JAK2*$^{V617F}$, 3 *CALR*$^{MUT}$, and 5 triple negative.
‡Platelet count at diagnosis was unknown in 13 patients: 4 *JAK2*$^{V617F}$, 3 *CALR*$^{MUT}$, and 6 triple negative.
§The 1 patient with a mutant level >50% had post-ET myelofibrosis at the time of analysis.
¶Two-sided Fisher's exact test.

CCTCAGTC-3′) by heteroduplex analysis on the WAVE DNA Fragment Analysis System (Transgenomic Ltd., Glasgow, United Kingdom [UK]). Samples with abnormal chromatograms were directly sequenced. The relative *CALR* mutant level was quantified using the same polymerase chain reaction with a fluorescently labeled forward primer and analysis on the Beckman Coulter CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Mutant level was expressed as a relative proportion of total *CALR* alleles.

## Results and discussion

*CALR* mutations were detected in 26 patients (25%): 9 (35%) were type 1 (p.L367Tfs*46), 11 (42%) were type 2 (p.K385Nfs*47), and 6 (23%) had other mutations. In all cases except 1, the predicted mutant would give rise to the common novel C-terminal peptide.[16-18] The remaining patient had 2 mutations on the same allele (c.1137_1138insAG and 1227_1231del; p.E380Rfs*38) that replaced the final 20 amino acids of the mutant peptide with an alternative 7 amino acid sequence. In keeping with data reported by others,[16-19] *CALR*$^{MUT}$ patients were significantly younger at diagnosis ($P = .0002$) and had significantly higher platelet counts than *JAK2*$^{V617F}$ patients (n = 43; $P = .001$; Table 1). There was no difference in age or platelet count at diagnosis between *CALR*$^{MUT}$ and the *JAK2*/*MPL*/*CALR* triple-negative patients (n = 34; Table 1). Two patients (2%) were *MPL*$^{MUT}$ and were excluded from further analysis. Cytogenetics were available from 36 patients (19 *JAK2*$^{V617F}$, 9 *CALR*$^{MUT}$, and 8 triple negative); all were normal except for 1 *JAK2*$^{V617F}$-mutated patient with trisomy chromosome 8 and chromosome 9.

XCIP analysis could be interpreted in 61 of the 105 female patients (58%): 16 *CALR*$^{MUT}$, 23 *JAK2*$^{V617F}$, and 22 triple negative. Excluded patients either lacked a polymorphic marker (n = 6), had constitutional or age-related skewing (n = 36), or were *MPL*$^{MUT}$ (n = 2). There was a strong correlation between a clonal XCIP and presence of a *CALR* mutation: 14 (88%) of *CALR*$^{MUT}$ patients were clonal, 5 of 6 interpretable cases with a type 1 mutation, and 6 of 7 cases with a type 2 mutation. This was significantly different from *JAK2*$^{V617F}$ (26%; $P = .0002$) and triple-negative patients (9%; $P < .0001$; Table 1). The difference was not related to the length of time from diagnosis at first test, which did not differ between *JAK2*$^{V617F}$ and *CALR*$^{MUT}$ patients ($P = .5$). The *CALR* mutant level for all patients was significantly higher than the *JAK2*$^{V617F}$ level, both in samples available at or within 3 months of diagnosis (median,

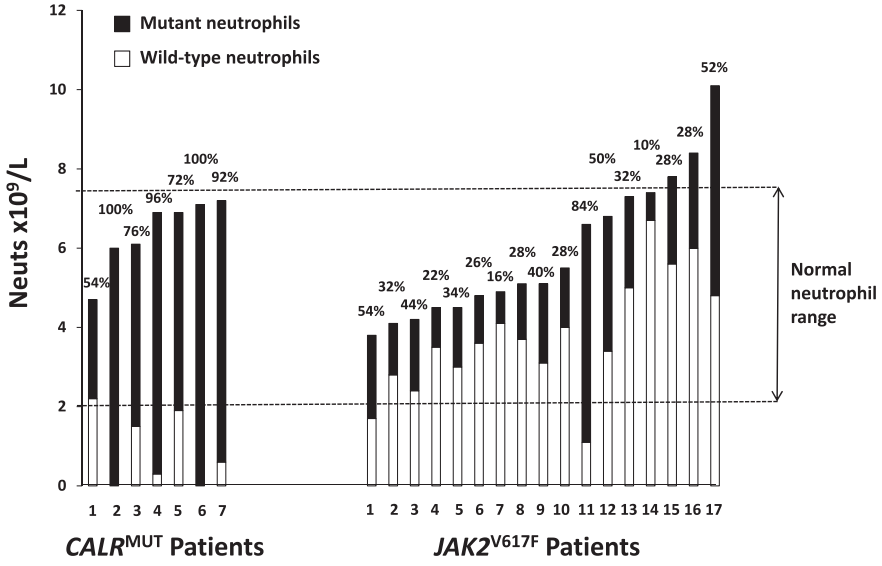


**Figure 1. Absolute number and relative proportion of wild-type and mutant neutrophils in *CALR*$^{MUT}$ and *JAK2*$^{V617F}$ ET patients tested prior to receiving any cytoreductive therapy.** Each bar represents an individual patient and shows the total neutrophil count at the time of testing, as well as the derived absolute number of mutant-positive and wild-type neutrophils. The latter are calculated from the relative proportion of mutant-positive neutrophils, as given above the bar, and assumes that mutant-positive cells were heterozygous for the mutation (ie, the proportion of mutated cells is double the percentage of mutant alleles).

46% vs 17%; $P < .0001$) and overall (median, 50% vs 18%; $P < .0001$; Table 1). No change in mutant level was observed in serial samples from 9 *CALR*$^{MUT}$ patients over 3 to 116 months of follow-up. The median difference was 1% (range, 0-15%), irrespective of treatment. Unlike the situation in *JAK2*$^{V617F}$ cases, this is therefore consistent with the presence of a heterozygous mutation in the majority of cells. This is supported by the XCIP data where 12 of the 14 clonal *CALR*$^{MUT}$ patients had monoclonal hematopoiesis with ≥95% expression of 1 allele. One of the 2 *CALR*$^{MUT}$ patients with a polyclonal XCIP had a lower mutant level of 30% with a borderline constitutively skewed XCIP (85%:15% expression in neutrophils, 72%:28% in T cells), and the observed neutrophil XCIP was only 4% different from the expected pattern of 89%:11% if the mutant-positive clone expressed the predominant X allele. This could not explain the polyclonal pattern in the remaining *CALR*$^{MUT}$ patient (42%:58% expression in neutrophils, 50%:50% in T cells; mutant level 36%). It is possible that this case is truly biclonal, as reported in a proportion of myeloproliferative neoplasm patients,[21,22] with 2 major clones expressing different X alleles.

*CALR*$^{MUT}$ patients have significantly lower white cell counts than *JAK2*$^{V617F}$ patients,[16,18,19] and immunohistochemistry studies have suggested that mutant CALR predominantly affects just the megakaryocytic lineage, with little/no expression in mature myeloid cells.[23] To further investigate the difference in myelopoiesis, we used the mutant allele level (assuming heterozygosity) to calculate the absolute number of mutant and wild-type neutrophils in 24 mutant-positive patients tested prior to receiving any cytoreductive therapy. A *CALR* wild-type neutrophil count in the normal range for our institution (2.0-7.5 × $10^9$/L) was observed in only 1 of 7 *CALR*$^{MUT}$ patients compared with 15 of 17 *JAK2*$^{V617F}$ patients ($P = .001$; Figure 1), which is indicative of suppression of wild-type myeloid cells at the hematopoietic stem cell stage in the *CALR*$^{MUT}$ but not *JAK2*$^{V617F}$ patients.

These results provide further evidence of the impact of *CALR* mutations on the hematopoietic stem cell with expansion of a dominant mutant-carrying clone, which leads to a distinct subtype of ET with a markedly different biology from that associated with *JAK2*$^{V617F}$ mutations.

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## Authorship

Contribution: R.E.G. and D.C.L. designed the study; C.A., J.R.L., and R.E.G. performed experimental work and analyzed the data; and R.E.G. and D.C.L. wrote the manuscript, which was reviewed by all authors.

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