Calreticulin gene exon 9 frameshift mutations in patients with thrombocythosis

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Somatic frameshift mutations in exon 9 of the calreticulin (CALR) gene were recently identified in patients with BCR-ABL-negative myeloproliferative neoplasms (MPNs), particularly essential thrombocythemia and myelofibrosis.1,2 Calreticulin is a highly conserved endoplasmic reticulum (ER) luminal Ca2+-binding chaperone protein with a critical role in the process of glycoprotein folding and a number of other cellular functions both inside and outside the ER,3,4 and has three domains with different structural and functional properties; a globular N domain, a proline-rich P domain and an acidic C domain.3,4 The disrupted C-terminal region contains a KDEL ER-retention sequence multiple sites with high capacity for Ca2+ binding and sites for binding to the cell surface and to blood clotting factors.5,6

The specific role of calreticulin in thrombopoiesis is currently unknown and it is thus not clear how CALR mutations may drive MPN. However, some of the functions ascribed to calreticulin offer themselves as candidates. For example, as a Ca2+-sensor calreticulin regulates Ca2+ homeostasis and the ER stress response, which is important for megakaryocyte maturation and platelet formation.7 Interactions of the calreticulin N domain with thrombospondin-1 and LRPI mediate focal adhesion disassembly,8 keratinocyte and fibroblast migration9 as well as the expression of TGFβ3 and collagen.8 Collectively, these processes are related to wound healing and fibrosis, a hallmark of MPN pathology. Furthermore, calreticulin is expressed on the surface of human platelets and binds αβ integrin and glycoprotein VI to mediate platelet–collagen interactions.10 Finally, Ca2+ binding to the C domain of calreticulin regulates protein–protein interactions with other chaperones such as ERp57, which functions as a STAT3 inhibitor, this effect being enhanced by ER luminal complex formation between ERp57 and calreticulin as are other chaperone functions of ERp57.11 Alterations in the Ca2+-binding ability of this region could potentially disrupt interactions between the two chaperones and modulate or abrogate the STAT3 inhibitory mechanism. In support of this hypothesis, activation of STAT3 has been shown in a number of essential thrombocythemia patients that lack JAK2 mutations,13 whereas Ba/F3 cells expressing mutant CALR mutations had, on average, higher hemoglobin concentration and sequence context suggests that they have a role in the generation of CALR mutations by illegitimate local recombination associated with deletions, insertions and inversions (Figure 2a). The second most common mutation was a 5-bp insertion (TTGTC) after position nt1127 of the cDNA found in seven cases. Interestingly, these and all other mutations identified reside within a repetitive region associated with deletions, insertions and inversions (Figure 2a). Of 289 samples tested, 189 (65%) carried a JAK2 V617F mutation and 8 (3%) an MPL codon 515 mutation (7 W515L and 1 W515K). Of the remaining 92 samples, 25 were found to carry a CALR exon 9 indel mutation (Figure 1a). Patients with JAK2 V617F mutations had, on average, higher hemoglobin concentration compared with patients with thrombocythemia and none of the three mutations studied. Also, patients with either JAK2 V617F or CALR exon 9 mutations had significantly higher platelet counts than patients with thrombocythosis and no mutations (Figure 1b).

Each of the 25 CALR mutant samples was found to harbor one of seven different indels; all leading to a +1 frameshift of the open reading frame, including two that have not been previously described. The most common mutation, found in 13 out of the 25 cases, was a 52-bp deletion of nt1172 to nt1223 of the CALR complementary DNA (cDNA; NM_004343.3). The second most common mutation was a 5-bp insertion (TTGTC) after position nt1127 of the cDNA found in seven cases. Interestingly, these and all other mutations identified reside within a repetitive region containing two simple and three tandem repeats whose location and sequence context suggests that they have a role in the generation of CALR mutations by illegitimate local recombination associated with deletions, insertions and inversions (Figure 2a). In particular, the TTGTC, which starts at the end of the second simple repeat, whereas the 52-bp deletion occurs between two GCGAGG heptanucleotide stretches (nt1092_1098 and nt1143_1150) (Figure 2a). One novel variant involved a complex frameshift of 5-bp (Figure 1b).
13 bp deletion and an inversion insertion of an AGACAA sequence, complementary to part of the deleted 13 bp. Another patient with a 31-bp deletion appeared to also carry a constitutional change of nt1145C > G such that the mutant protein sequence differed from any of those previously described (Figure 2b). Interestingly, the mutation hotspot contains a stretch of 38 nucleotides without
a or T (such a stretch is expected to occur every $2.7 \times 10^{11}$ nucleotides assuming random distribution of A, C, G, T). As observed by others, all CALR exon 9 mutations identified were associated with loss of the C-terminal KDEL moieties and led to the generation of a novel peptide sequence terminating with the same 36 amino acids. Crucially, this alteration transforms the negatively charged glutamic-acid-rich C terminus of calreticulin to a positively charged arginine-rich region (Figure 2b), and this may have a crucial role in mediating the effects of these mutants.

The assay we describe here employs primers situated outside the CALR genomic region affected by any of the indel mutations described so far, and this makes it a useful diagnostic tool in the investigation of thrombocytosis or other findings suggestive of MPN. Most mutations were easily detectable by standard PCR and agarose gel electrophoresis because of the formation of a prominent heteroduplex band migrating slower than the wild-type product in the gel. However, in two of the patients carrying a much lower mutation burden than the rest of the patients, the mutations were not detectable on agarose gel electrophoresis. By contrast, all mutations were detected by PCR followed by capillary gel electrophoresis and fragment analysis (Figure 2c). To determine the sensitivity of PCR fragment analysis, we serially diluted five samples carrying different indel mutations with control DNA. These samples had an estimated mutation burden between 40% and 50% based on relative peak areas of the mutant and wild-type PCR products. In all five samples we were consistently able to detect the mutation after a 1:10 dilution, giving this assay a sensitivity to a mutant allele burden of 5% or less (e.g. Figure 2d).

Altogether, JAK2 V617F, CALR exon 9 indel and MPL codon W515 mutations were found in 77% of the patients referred to our laboratory for the investigation of persistent thrombocytosis. We presume that the remaining 23% of the patients either had secondary thrombocytosis or a clonal disorder driven by rare unknown mutations. The high incidence of CALR indel mutations in patients with persistent thrombocytosis suggests that CALR mutational screening should be included in the routine mutational screening should be included in the routine investigation of persistent thrombocytosis, even before strict criteria for the diagnosis of MPN have been established. The method described here, although simple and easy to perform, has the ability to cover the wide range of CALR exon 9 mutations and is sensitive enough to detect low mutation burdens. Also, the identification of previously undiscovered CALR exon 9 mutation variants, while giving further validity to our assay, suggests that more such variants are likely to be discovered in MPN patients in the future, making the use of such a generic assay more important.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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