Review Article

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Clinical relevance between CALR mutation and myeloproliferative neoplasms

Zhiyuan Wu[1]*, Chen Zhang[1]*, Xiaochao Ma[3], Ming Guan[1,2]

[1]Department of Laboratory Medicine, Huashan Hospital North, Shanghai Medical College, Fudan University, Shanghai 201907, China; [2]Central Laboratory, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China; [3]Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15260, USA

*These authors contributed equally to this work.

Correspondence to: Ming Guan. Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China. Email: guanming88@yahoo.com.

Abstract: In late 2013, somatic mutations in calreticulin (CALR), mainly those involving insertions and deletions in exon 9, attracted the great attention of hematologists and researchers. These JAK2- and MPL-mutual exclusive mutations enjoy a favorable specificity and prevalence (20-30%) in patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), suggesting promise for these mutations in disease management. Moreover, these genetic variations are now also considered as a group of independent risk factors for disease prognosis. In this mini-review, we will document the value of CALR mutations in disease diagnosis, prognosis, and therapeutic strategy selection, and we will discuss current advances in methods to detect these mutations.

Keywords: Calreticulin (CALR); genetic mutation; myeloproliferative neoplasms

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Identification of CALR in myeloproliferative neoplasms (MPNs)

Calreticulin (CALR) was originally identified as a Ca^{2+} binding protein in the endoplasmic reticulum lumen of most cells of human origin. Its main function is to play a critical role in quality control processes during protein synthesis and folding, through binding to misfolded proteins. CALR is found at multiple subcellular localizations outside of the endoplasmic reticulum, where it mediates a variety of cellular processes, including apoptotic cell clearance, cell adhesion, and cell migration (1-3). Moreover, CALR is implicated in a variety of cellular roles, including modulation of activation of the unfolded protein response (UPR) and calcium signaling, Ca^{2+} storage, regulation of steroid-sensitive gene expression, cell adhesion, chaperoning in protein folding, autoimmune response, and neuromodulations (4,5).

In December 2013, Klampfl *et al*. (6) and Nangalia *et al*. (7) identified somatic mutations in exon 9 of CALR as the second most prevalent acquired nucleotide changes in JAK2 mutation-negative essential thrombocythosis (ET) and primary myelofibrosis (PMF) patients. But these are not found in patients with polycythaemia vera (PV). Klampfl *et al*. (6) adopted whole-exome sequencing to analyze genomic DNA from granulocytes and T-lymphocytes from six patients with PMF. This approach led to the identification of somatic mutations of CALR, the gene encoding CALR, in all six patients. At the same time, Nangalia *et al*. (7) performed whole-exome sequencing in 151 patients with MPNs. They found CALR exon 9 mutations in most subjects with non-mutated JAK2. CALR mutations are mutually exclusive, with mutations in both JAK2 and MPL, and are found in about 20% to 25% of all patients with ET or PMF.

All CALR mutations are insertions or deletions resulting in a frameshift, and cluster in the last exon (exon 9) of the gene. Thus far, more than 50 different types of mutations in

CALR have been detected. Two specific mutations are most prominent, a 52-bp deletion (type 1 mutation) and a 5-bp insertion (type 2). Overall, these two mutation types are found in more than 80% of all patients with mutant CALR.

Diagnosis

Somatic mutations in CALR, after those in JAK2, are the second most prevailing genetic variation in ET and PMF. The first two reports identified and validated the high prevalence of CALR somatic mutations in JAK2 and MPL double-negative ET and PMF patients' granulocyte DNA (6,7), indicating that approximately 90% of patients with ET or PMF harbor the mutually exclusive mutations in JAK2 (50-60%), CALR (20-30%), or MPL (5-10%). Although these variants consist of over 50 types of insertions and deletions in the exon 9 of CALR (8), they do not present in lymphoid cancer, solid tumors, and normal healthy controls, showing a favorable specificity to ET and PMF and holding promise as an ideal marker for the molecular diagnosis of disease.

In patients harboring the clinical indicators for the diagnosis of ET, the presence of mutations in CALR may serve as an optimal clonal marker to rule out the reactive thrombocythemia, since the differential diagnosis may be difficult without molecular evidence (9). Given the encouraging specificity and sensitivity to the disease of CALR mutation, genetic screening for CALR exon 9 can notably lead to revision of the current diagnostic route for ET and PMF, and will undoubtedly be embedded in front-line tests of MPNs and World Health Organization diagnostic criteria for these disorders (10).

However, several recent observations have reported the co-occurrence of JAK2 and CALR mutations, not only in ET (11-13) and PMF (14) patients, but also in some PV cases (15). It is worth noting that indels in CALR exon 9 have also been reported in a JAK2 mutation-negative PV patient (16). Two conclusions could be drawn from these findings. A positive result for CALR mutation screening, while demonstrating a favorable specificity for ET and PMF diagnosis, cannot be used as the exclusive marker for PV. On the other hand, JAK2 and CALR mutations, as the most prevalent genetic alterations of Ph-negative MPNs, may contribute to the diversity of the disease pathogenesis and development.

Clinical features and prognosis

Genetic impairment of JAK2, MPL, and CALR, the three major driver mutations of classical Ph-negative MPNs, affects the molecular background of these hematopoietic abnormalities and can lead to a distinct pathological phenotype. In general, ET and PMF patients with mutant CALR exon 9 will present a prognostic advantage over those without CALR mutation, as survival can be favorably affected by CALR variants (14,17).

In ET, patients with CALR mutations have been reported to have an earlier disease onset, higher platelet count and serum erythropoietin, lower white blood cell count and hemoglobin level compared with those with JAK2 V617F, indicating that CALR mutation could favor megakaryocytic cell expansion, while JAK2 V617F favors erythropoiesis. Moreover, these mutations show a gender preference, as male patients are more vulnerable to CALR mutation (13,17,18). Reduced thrombosis risk has been observed widely in CALR-mutated or JAK2-/MPL-/CALR- triple negative patients, compared to those with JAK2 V617F (19). However, it is worth underlining a recent large scale study from Italy involving 1150 ET patients reported CALR mutation may not contribute as an independent risk factor for the international prognostic score for the risk of thrombosis (IPSET) thrombosis risk evaluation, though they also observed that CALR mutation was more frequently distributed in the low- and intermediate-risk groups than in the high-risk group (20). Although no evidence exists to support the hypothesis that CALR mutation is responsible for the difference in the overall survival in ET (21), the thrombosis-free survival in CALR mutant or triple-negative patients was significantly better than that of patients with the JAK2 or MPL mutation (13,17).

In PMF, CALR exon 9 mutations are associated with younger morbidity age, higher platelet count and hemoglobin level, lower leukocyte count, and reduced incidence of spliceosome mutations. Patients with CALR mutation are also inclined to present a lower score on the Dynamic International Prognostic Scoring System-plus (DIPSS-plus) and are less likely to be anemic or require transfusions. With regard to the survival of disease, CALR mutations show a more satisfying effect on the overall survival apart from the DIPSS-plus risk stratification, compared with the JAK2 mutation or JAK2-/MPL-/CALR- triple negative profile, but they show a similar effect on survival as compared to MPL mutation. It is worth noting that triple-negative PMF patients not only suffer the worst outcome of overall survival but also display an inferior leukemia-free survival among all the genetic variants (14). Besides the highly prevalent JAK2 and

CALR mutations that contributes to the hematopoiesis and myeloproliferation phenotypes (22), recent studies also identified subclonal mutations in ASXL1, SRSF2, EZH2, IDH1 and IDH2 that lead to disease progression and leukemic transformation. Somatic mutation in ASXL1 is another independent risk factor for DIPSS-plus (23). Tefferi *et al*. have recently reported the collaboration between CALR and ASXL1 mutations in the prognosis of PMF, emphasizing an association between prolonged survival and the CALR+/ASXL1- variation pattern. According to the data, PMF patients harboring CALR+/ASXL1-profile showed a prolonged survival of over 8 years, compared with those with CALR-/ASXL+, while CALR+ASXL1+ and CALR–ASXL1– patients were in a similar intermediate risk category (24).

In addition, emerging literature suggests the prognostic advantage of CALR mutations in ET and PMF could be ascribed to type 1 or type 1-like variants (25). The type 1 mutation, as opposed to the type 2 CALR variant, has been reported to bring about lower platelet count in ET (19) and prolonged survival in PMF (26). According to the results from bioinformatic analyses, the 5-base pair insertion generates a higher C terminus α-helix in the type 2 variant, which is similar to that of the wild-type CALR, in comparison with the lower helix content of the type 1 variant (27).

Treatment

Lately, an integrated genomic analysis of MPNs confirmed the central role of JAK-STAT pathway activation in the pathogenesis of the diseases. The gene expression profile characterized the increased JAK2 activity in MPNs patients, regardless of the JAK2 or CALR mutation status (28). The consistent activated JAK-STAT pathway also gave a promising vision for the JAK inhibitor treatment for the CALR mutant MPNs. However, the currently available drug therapy for PMF still does not offer a satisfying strategy to alleviate bone marrow impairment and prolong survival (29,30). To address this problem, Tefferi *et al*. suggested that asymptomatic patients with low or DIPSS-plus intermediate-1 risk or low risk mutation profiles such as CALR+/ASXL1- be observed without any therapeutic intervention or be managed by conventional drug therapy, while those patients with DIPSS-plus intermediate-2 or high risk or unfavorable genetic profile such as CALR-/ASXL1+ be subjected to transplant or investigational drug therapy (31). Immuno-therapy is one of the classical therapeutic alternations for MPNs. As inspired by wild-type CALR-induced interferon alpha resistance in patients with hepatitis B virus infection (32), Cassinat *et al*. have recently described the positive effect of pegintereron alfa-2a in two young CALR mutant ET patients, which resulted in a prolonged hematologic complete response (33). This finding also suggested a potential therapeutic target of the CALR mutant MPNs.

Current status and prospects for CALR mutation detection and monitoring

Molecular pathology plays an important role in the diagnosis of ET and PMF. Although the well-known mutation in JAK2 exon 14 and MPL exon 10 has generally been accepted as the diagnostic marker for approximately 60% of ET and PMF cases, genetic variation in CALR exon 9 can still be detected in 60-70% patients lacking these JAK2 and MPL mutations (6,7). The high prevalence and specificity of CALR mutation in ET and PMF implicates the irreplaceable diagnostic value of detecting the mutation.

Thus far, over 50 types of documented insertions and deletions in CALR exon 9 have been identified that can lead to gene open read frame shifting. The high alterability of these genetic variations impels long-range mutation scanning to be the only general method to assess all the potential variants. The currently available technologies to identify and characterize CALR exon 9 mutations are mainly focused on sequencing and fragment analysis. Unfortunately, few of them have been literally reported, except for the time-honored Sanger sequencing strategy and high resolution melting analysis (HRMA). Sanger sequencing is currently the most available strategy for gene sequence capture and analysis. Since the CALR mutations have the heterozygote predisposition of showing a burden of 35-50% (18,34), the inconspicuous sensitivity of the method did not impede it from becoming the most favored approach for mutation identification and characterization (13). However, Sanger sequencing, owing to its fair sensitivity of 15-20% mutation load, can still cause false-negative results in identifying the CALR mutation in some rare cases (35). In these cases, full-coamplification at lower denaturation temperature (COLD) PCR (36) can be adopted to the amplification of target fragment for sequencing, so as to bring a more favorable detecting sensitivity.

However, the process of Sanger sequencing is demanding and labor intensive, encouraging the emergence of such fast and convenient methods as HRMA and gel capillary

Table 1 Frequency of common mutations in ET and PMF patients

Study	JAK2 (%)	MPL (%)	CALR (%)	Triple-negative (%)	Disease	Population
Tefferi *et al*. (19)	57	3	28	12	ET	North America (United States)
Tefferi *et al*. (26)	58	8.3	25	8.7	PMF	North America (United States)
Rumi *et al*. (42)	64.7	4	22.7	8.6	PMF	Europe (Italy/Spain)
Rotunno *et al*. (17)	64.1	4.3	15.5	16.1	ET	Europe (Italy)
Chi *et al*. (34)	65	3	8.7	23.3	ET	Europe (Cyprus)
Wojtaszewska *et al*. (43)	61	1	13	25	ET	Eastern Europe (Poland)
Wu *et al*. (40)	56.3/58	5/6	25/32	13.7/4	ET/PMF	East Asian (Mainland China)
Li *et al*. (38)	50	3	21	26	PMF	East Asian (Mainland China)
Chen *et al*. (13)	63.9	2.7	22.5	10.9	ET	East Asian (Taiwan China)

ET, essential thrombocythemia; PMF, primary myelofibrosis; CALR, calreticulin.

electrophoresis, though they cannot explicitly specify the exact genotype of the mutation.

As introduced by Wittwer *et al*. (37) in 2003, HRMA is now the preferred closed tube method for small indels. Several methods based on HRMA have been reported (8,35) with a sensitivity of 3-5% mutation load and a turnaround time of 1 hour for the experimental procedure. By fitting the plotting of the melting curve with a standard genotype control, researchers can also acquire the preliminary genetic characteristics of each variant. Aside from HRMA, the 6-FAM-labeled forward primer (34) is more handy than Sanger sequencing and more straightforward than HRMA in interpreting results.

Among the more than 50 most prevalent frame-shift mutations in CALR exon 9, the 52-base pair deletion and 5-base pair insertion are identified as the predominant mutation types, accounting for more than 80% of the mutations (6,7), though the prevalence can vary between different ethnicities (38). Meanwhile, the type 1 and type 2 CALR mutation can lead to a diverse clinical phenotype and prognosis (25), again revealing the importance of the need for definite genotyping of CALR mutations. As for these two specific mutations, allele specific methods such as Taqman real-time PCR can be utilized to identify mutations since they enjoy favorable specificity and sensitivity. As described by Chi *et al*. (8), the qPCR method can discriminate both the type 1 and type 2 mutations at a sensitivity of 1% mutation load.

Although the currently available assays have mostly satisfied the need for the identification of CALR mutation, development of other fast fragment analysis approaches, such as denaturing high performance liquid chromatography, should still be encouraged to enrich the alternatives for mutation detection. On the other hand, we believe that cutting-edge methods such as high-throughput target re-sequencing strategy for genome scanning of JAK2, CALR, MPL, LNK, TET2, ASXL1, IDH1/IDH2, EZH2, DNMT3A, CBL, TP53, SRSF2, SF3B1, and U2AF1 will radically improve the diagnostic and management algorithm of ET and PMF.

Distribution of CALR mutation and type in different ethnicities

Previous literature has described the difference in the prevalence of JAK2 mutant PV patients in diverse populations (39-41). However, in ET and PMF patients the estimated occurrence of the three major mutant genes, JAK2, MPL, and CALR mutations turned out to be conservative (*Table 1*). CALR mutation, in most cases, occurred in over half of JAK2- and MPL- negative ET and PMF patients, suggesting the distinctive pathological value of these genetic alterations.

In the light of the currently available literature (*Table 1*), indel mutations in CALR exon 9 occur with a similar frequency in both ET and PMF patients regardless of ethnic diversity. Although the reports from Rotunno *et al*. (17), Chi *et al*. (34), and Wojtaszewska *et al*. (43) suggested a lower occurrence of CALR mutation in the European population, this difference could be attributed to the limited sample scale and their strict obedience to the WHO criteria.

The discovery of mutations in CALR exon 9, as the complement of JAK2 and MPL genetic alterations, further filled the gap in knowledge about the genetic background of classical MPNs. Emerging studies are focusing on

the mutation distribution in diseases, establishing the connection between mutation and disease phenotypes, and developing mutation scanning methods with improved sensitivity and operability. However, deeper understanding of these genetic variations has also prompted clinical practitioners and researchers to devote themselves to the study of these mutations at the molecular pathology level, to optimize the molecular diagnostic algorithm, to clarify the pathogenic mechanism of the mutations, and to locate the therapeutic target of the impaired signaling pathway.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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