Calreticulin (CALR) mutation in myeloproliferative neoplasms (MPNs)

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Abstract: As a heterogeneous group of disease, myeloproliferative neoplasms (MPNs) have confused hematologists and hematopathologists with their protean clinical presentations and myriads of morphologies. A thought of classifying MPNs based on molecular alterations has gained popularity because there is increasing evidence that molecular or chromosomal alterations have a better correlation with clinical presentation, response to therapies, and prognosis than conventional morphological classification. This type of efforts has been facilitated by the advancement of molecular technologies. A significant number of gene mutations have been identified in MPNs with JAK2 and MPL being the major ones. However, a significant gap is present in that many cases of MPNs do not harbor any of these mutations. This gap is recently filled by the discovery of Calreticulin (CALR) mutation in MPNs without JAK2 or MPL mutation and since then, the clinical and molecular correlation in MPNs has become a hot research topic. There seems to be a fairly consistent correlation between CALR mutation and certain hematological parameters such as a high platelet count and a better prognosis in MPNs with CALR mutation. However, controversies are present regarding the risks of thrombosis, interactions of CALR with other gene mutation, the role of CALR in the pathogenesis, and the optimal treatment strategies. In addition, there are many questions remain to be answered, which all boiled down to the molecular mechanisms by which CALR causes or contributes to MPNs. Here, we summarized current published literatures on CALR mutations in MPNs with an emphasis on the clinical-molecular correlation. We also discussed the controversies and questions remain to be answered.

Keywords: Myeloproliferative neoplasms (MNPs); Calreticulin (CALR); clinical correlation

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Introduction

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of chronic myeloid neoplasms that have the potential to progress to acute leukemia. This group of neoplasms is best exemplified by the chronic myeloid leukemia (CML) associated with Philadelphia chromosome/BCR-ABL1 fusion gene. Other common entities in this group include polycythemia vera (PV), essential thrombocythemia (ET), and primary (idiopathic) myelofibrosis (PMF). CML, PV, ET, and PMF were first described in 1951 by William Dameshek as “myeloproliferative disorders”, accordingly, they are now referred to as “classic” MPNs, and PV, ET, and PMF are referred to as classic BCR-ABL1-negative MPNs (WHO classification 2008). PV, ET, and PMF are stem cell–derived clonal disorders and have been associated with various gene mutations, including JAK2 (1), MPL (2), TET2 (3), ASXL1 (4), IDH1 (5), IDH2 (6), CBL (7),
IKZF1 (8), LNK (9), EZH2 (10), DNMT3A (11), and CALR (12,13). Of which, JAK2 and MPL mutations appear to exert a phenotype-modifying effect and are distinctly associated with PV, ET, and PMF, with a high frequency (99%, 55% and 65% for JAK2 and 0, 3% and 10% for MPL mutations). Calreticulin (CALR) gene was first recognized as a somatic mutation in patients with MPNs who had no mutations in either JAK2 or MPL by Klampfl et al. (13) and Nangalia et al. (12) in 2013. Ever since this discovery, its molecular and pathogenic roles, and its clinical significance have been the hot research topic in the field of hematopathology. It has been proposed to incorporate CALR mutation status in 2014 revision for WHO diagnostic criteria for BCR-ABL1-negative MPNs (14). This review is to summary current published data on CALR in relation to MPNs.

**CALR and types of CALR gene mutations**

The CALR gene is located in the short arm of chromosome 19 (19p13.2). It contains 9 exons and spans 4.2 kb (http://omim.org/entry/109091). All the mutations identified so far occur in exon 9. With the exception of a few non-recurrent point mutations (15), almost all these mutations are indels. More than 50 different CALR indels have been described. Klampfl et al. has defined those with a 52-bp deletion (p.L367fs*46) as type 1 mutation and those with a 5-bp TTGTC insertion (p.K385fs*47) as type 2 mutations. They have found that these two types of mutations accounted for more than 80% of all CALR-mutant patients and the other mutation types including type 3, 4, 5, and 11 were observed at much lower frequencies, many of which were detected only in a single patient (13,16). Interestingly, the frequency of type 1 mutation is significantly higher in PMF than in ET, suggesting a specific role of this mutation in myelofibrotic transformation (17,18). Most of the mutations are heterozygous mutation. Homozygous CALR mutations are very rare and they are all 5-bp insertions (12,13).

It was found initially that CALR and JAK2 mutations were mutually exclusive. However, CALR and JAK2 co-mutations have been reported recently in a few MPN cases across different ethnic groups, although the frequency of co-mutation is usually below 1% (19), including rare cases of refractory anemia with ring sideroblasts and thrombocytosis (20), PMF (21), and ET (22,23). Most molecular techniques involving CALR mutation testing are Sanger sequencing and polymerase chain reaction (PCR) followed by fragment analysis (12,13). Using TA cloning and high resolution melting (HRM) analysis, Lim et al. found higher CALR mutation rate than using Sanger sequencing; 12 out of 14 ET patients with wild type CALR by Sanger sequencing are positive for CALR mutation by TA cloning and HRM analysis, suggestive of low-allele-burden CALR mutants in these patients. In the same study, Lim et al. also demonstrated co-existence of CALR exon 9 alteration and JAK2 mutations in 13 (22%) of their 59 JAK2 mutated ET cases, suggesting that CALR exon 9 mutations in the presence of JAK2 mutation might be far more common than they were thought and that the undetectable CALR mutation in the background of JAK2 mutation was mostly likely due to a low allele burden which is below the detection limits of commonly used technique (24).

Majority cases of CALR mutations in MPNs were type 1 and type 2 that caused a frame shift to the alternative reading frame 1. As a result, mutant CALR proteins contains a number of positively charged amino acids (type 1 eliminated almost all negatively charged amino acids and type 2 retained approximately half the negatively charged amino acids), which has been hypothesized that the mutations types of CALR may be associated with qualitatively different phenotypes of MPNs (13). CALR point mutations (E381A and D373M) and inframe deletions (E381_A382>A, D397_D400>D, D400_K401>D and E405_V409>V) were also detected in patients with suspected MPNs and JAK2-mutated MPNs in another study albeit with a lower frequency. These CALR alterations were also found to co-exist with other genes associated with MPNs such as MPL, CSF3R, ASXL1 and ZRSR2. Currently, the role of these CALR point mutations and inframe deletions in the molecular pathogenesis of MPNs is not yet clear. Because they frequently co-exist with mutations involving the JAK-STAT signaling pathway and affect disease phenotype in JAK2-mutated ET patients, these non-classic CALR mutant proteins are suspected to have a contributory role in the pathogenesis of MPNs (24).

Approximately 5-10% of MPNs are triple negative for JAK2, MPL, and CALR (25). Mutations in many other genes including ASXL1, DNMT3a, TET2, EZH2, IDH1, IDH2, as well as in genes involved in mRNA splicing, such as SF3B1 and U2AF2, have also been described in recent years in patients with MPNs (26). It is not clear whether they explain all the MPNs when all these mutations are taken into account. It is also not clear whether these mutations are mutually exclusive or whether they are the drivers of the disease. The possible interactions between these genes are discussed as follows.
Ethnic differences

Studies have shown that there are significant differences in the frequencies and the types of mutations between the Caucasian and Chinese MPN patients. Rumi et al., on behalf of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie (AGIMM), did investigation on 617 patients with PMF and found that of 617 patients, 399 (64.7%) carried JAK2 (V617F), 140 (22.7%) had a CALR exon 9 indel, 25 (4.0%) carried an MPL (W515) mutation, and 53 (8.6%) had non-mutated JAK2, CALR, and MPL (so-called triple-negative PMF) (17). Similar study conducted by Li et al. (27) on 357 Chinese patients with PMF and found that of 357 patients, 178 (50%) carried JAK2 (V617F), 76 (21%) had a CALR mutation, 11 (3%) carried an MPL mutation, and 96 (27%) had non-mutated JAK2, CALR, and MPL (so-called triple-negative PMF). This study showed a similarity in the frequency of CALR mutations between Chinese patients and European patients. The difference was, however, that they found 27% of Chinese patients were triple-negative, which is significantly higher than that of 8.6% in European patients by AGIMM study. Another study conducted by Rumi et al. on 745 European ET patients found that numbers (%) of patients with JAK2 mutation, CALR mutation, MPL mutation, or triple negative (non-mutated JAK2/CALR/MPL) were 466 (62%), 176 (24%), 28 (4%), and 75 (10%), respectively (28). Fu et al. also tested for CALR, JAK2, and MPL mutations on 436 Chinese ET patients and found that of 436 patients, 240 (55.1%) had JAK2 V617F mutation, 99 (22.7%) showed CALR mutation, 6 (1.4%) had MPL mutation, 89 (20.4%) had non-mutated JAK2, CALR, and MPL, and 2 (0.5%) patients harbored both JAK2 and CALR mutations (29). These two studies on ET patients also showed similar mutation rates for JAK2 and CALR mutations between Chinese and European ET patients, but much higher rate for triple-mutation in Chinese ET patients than European ET Patients. The ethnic differences would imply that currently unknown mutant genes are responsible for ET or PMF in a considerable proportion of patients of Chinese descent. Another significant difference between the studies on PMF patients conducted by Rumi (17) and Li et al. (27) is in the types of CALR mutations detected. In the Rumi study on Caucasian patients, 72% of CALR mutations are 52-bp deletion (type 1 mutation), 16% are 5-bp insertion (type 2 mutation), and the remaining 12% are other type less frequent indels. A recent French study (18) also showed over-representation of type-1 CALR mutation (70%) and under-representation of type-2 CALR mutation (13%) in PMF as compared with ET. In the study by Li et al. by contrast, type-1 mutation was found 32% and type-2 mutation in 64% of the PMF patients studied (25).

CALR mutation may have different prognostic value in different ethnicities. In a Korean study, ET patients carrying CALR mutation were younger, had lower white blood cell counts, and experienced less thrombosis during follow-up than those carrying JAK2 V617F mutation, while both patient groups showed similar clinical features and prognosis, without significant difference in hemoglobin level and platelet count. When JAK2 mutation is negative, CALR mutation had no influence on clinical and prognosis in ET patients. CALR mutation analysis could be a useful diagnostic tool for ET and PMF in 50% of the cases without JAK2 V617F mutations, although the prognostic impact of CALR mutations needs further investigation (19).

CALR in other disease

CALR mutations identified in MPNs so far are all somatic mutations since they are only present in granulocytes but not lymphocytes (30). Somatic CALR mutations have been rarely identified in patients with other disorders including Budd-Chiari Syndrome and Portal Vein Thrombosis (31). Germine mutations are even rarer and have only been identified in Schizoaffective disorder (32). Although CALR has long been implicated in the pathogenesis of many malignancies, it has not been demonstrated in any other malignancies other than myeloid cancers (33). CALR mutation has been detected in 8% of myelodysplasia samples, in occasional samples of other myeloid cancers, and in none of the other cancers (12).

CALR, driver or not?

Although CALR mutations are somatic, a particular germline mutation could synergize with JAK2 and CALR mutation in the initiation of MPNs based on the studies of 12 pedigrees with familial MPNs. This is based on the autosomal dominant pattern of the predisposition to develop MPNs (34). It has been suggested that CALR mutation occurs in the early phase of the disease. Clonal analyses showed CALR mutations in the earliest phylogenetic node, a finding consistent with its role as an initiating mutation in some patients (12). CALR mutations could be identified in the hematopoietic stem cell and progenitor cell compartments, consistent with an early event in MPN
pathogenesis (12,13). Klampfl et al. (13) showed that in two PMF patients with multiple mutations, the CALR mutations were early events, that is, they had already been present in the proliferative phase of PMF. In another case report, CALR mutation was an early genetic event and BCR-ABL1 was a secondary event leading to a clinically unusual MPN in this patient (35). Clonal analysis of the mononuclear cell colonies of two patients with MPNs demonstrated that CALR mutations were acquired first and were present in all colonies examined (22). An indirect evidence suggesting that JAK2, CALR, or MPL mutations are driver mutations is that these mutations define distinct disease entities within PMF, which have characteristic clinical presentations and prognoses (17). Overexpression of the most frequent CALR deletion caused cytokine-independent growth in vitro, further supporting its role as a driver mutation (13).

It is not clear how the CALR mutations are generated. Different from JAK2 or MPL mutations, CALR mutations are mostly deletions or insertions. However, CALR point mutations have been identified and are present with or without indels, implying that these scattered point mutations could be the predecessors of the frameshifting alternation (15). Therefore, CALR and JAK2 may share similar mechanisms during the generation of point mutations. The mutagenesis in JAK2 (36) is likely transcription driven. It is reasonable to postulate that CALR mutations may arise in a similar manner. The presence of mutations of many genes involved in epigenetic modification suggests chromosomal instability could be responsible for the generation of these point mutations which function as mediator for more complicated genetic rearrangement and lead to more complicated genetic alterations such as insertion or deletion.

Interaction between drivers and modifiers

As in all the complicated diseases, there are driver mutations and modifier mutations in MPNs and they interact with each other. In PMF, there is a synergistic effect between ASXL1 and CALR: in an international study of 570 patients, survival was the longest in CALR+ASXL1- (median 10.4 years) and shortest in CALR-ASXL1+ patients [median 2.3 years, hazard ratio (HR) of 5.9]. However, the double positive or the double negative patients had similar survival with an intermediate risk (median survival 5.8 years, HR 2.5). The CALR/ASXL1 mutations-based prognostic model was DIPSS-plus independent (P<0.0001) and effective in identifying low-/intermediate-1-risk patients with shorter (median, 4 years) or longer (median 20 years) survival and high-/intermediate-2-risk patients with shorter (median, 2.3 years) survival. Multivariable analysis distinguished CALR-ASXL1+ mutational status as the most significant risk factor for survival: HR 3.7 vs. 2.8 for age >65 years vs. 2.7 for unfavorable karyotype. These observations signify immediate clinical relevance and warrant (I) CALR and ASXL1 mutation determination in all patients with PMF and (II) molecular revision of DIPSS-plus. The possible mechanism is that CALR mutation is capable of handling the epigenetic alteration associated with ASXL1 (37). However, this study was contrasted by another study. In multivariable analysis, CALR mutations had a favorable impact on survival that was independent of both DIPSS-plus risk and ASXL1 mutation status (21).

In a study on familial MPNs, CALR mutation was found to be somatic. However, a particular germline mutation could synergize with JAK2 and CALR mutation in the initiation of MPNs based on the studies of 12 pedigrees with familial MPNs. This is based on the autosomal dominant pattern of the predisposition to develop MPNs (34). The interaction between CALR mutation and BCR-ABL1 gene has been demonstrated in an atypical MPN. In this case, CALR mutation was an early genetic event and BCR-ABL1 was a secondary event leading to a clinically unusual MPN in this patient. The del52CALR-positive, BCR-ABL1-positive subclone was sensitive to imatinib or dasatinib, but the del52CALR-positive, BCR-ABL1-negative clone remained resistant (35).

Mechanisms of CALR causing MPNs

CALR is a 46-kDa protein expressed in all cells of higher organisms. CALR was first purified and recognized as a Ca²⁺ binding protein of the skeletal muscle sarcoplasmic reticulum and was later hereditarily recognized in all mammals. CALR, a highly conserved protein, shows more than 90% amino acid sequence identity among the mammals, suggesting its important physiological functions. The CALR gene consists of nine exons and spans approximately 3.6 kb of human genomic DNA which is localized in chromosome 19. Structural predictions of CALR suggest that the protein has three domains; the N-domain, P-domain and C-domain (38). Although many mutations have been identified, they can be classified to type 1/type I-like and type 2/type 2-like based on their helix propensity for 31 unique amino acid sequences that were altered. Therefore, type 1 and type 2 mutations can serve as
CALR is a pleiotropic molecule that normally resides in the lumen of the endoplasmic reticulum (ER). It has various functions, ranging from regulation of calcium homeostasis to ensuring proper protein folding. More recently, CALR gained special interest for its extracellular functions, where it has direct immunomodulatory activity (39).

Not surprisingly, homozygous CALR knock-out mutation is embryonic lethal in mice. The death was due to severe problems of cardiac development (40). It makes it difficult to study the in vivo effect of CALR mutation. Overexpression of the most frequent CALR deletion caused cytokine-independent growth in vitro owing to the activation of signal transducer and activator of transcription 5 (STAT5) by means of an unknown mechanism (13).

All the mutations identified so far occur in exon 9. Exon 9 of CALR codes for the C-terminus of CALR protein which includes an ER retention signal. These mutations invariably generate a new C-terminus, which lead to the loss of ER KDEL retention signal. The fact that all the CALR mutations identified so far occur in exon 9 speaks for its significance in the pathogenesis. However, the intracellular distribution is not altered since mutant CALR was observed in the ER without increased cell-surface or Golgi accumulation (12).

However, in hematopoietic stem/progenitor cells, mutated CALR may not mediate the Ca$^{2+}$ export from the ER thus keeping the calcineurin-NFAT signaling pathway significantly less active, which in turn favors myeloid/megakaryocyte lineage commitment and not erythroid lineage proliferation. This may explain the observation that among the classical MPNs, CALR mutations are found only in PMF and ET and are not associated with erythrocytosis (41).

Activated JAK2 pathway may be a part of pathway (42). Notably, a transcriptional signature consistent with activated JAK2 signaling is seen in all MPN patients regardless of clinical phenotype or mutational status. In addition, the activated JAK2 signature was present in patients with somatic CALR mutations (42).

Other possible mechanisms based on the study of non-hematologic malignancies include reduced apoptosis and macrophage functions: CALR overexpression suppresses cell proliferation and enhances apoptosis on human MCF-7 breast cancer cells. The apoptosis is triggered by Calcium release from ER. Loss of CALR disables the cellular responses to apoptotic signals (38). CALR is expressed in many cancer cells but not most normal, stem or progenitor cells. It plays a role to promote macrophages to engulf hazardous cancerous cells. Therefore, the loss of CALR leads to decreased phagocytosis of cancer cells (43).

CALR is likely linked to MPNs through megakaryocytes, which has been reported to play a major role in MPN pathophysiology. CALR mutations resulted in higher number of megakaryocytic colonies than “triple negative” and JAK2 mutations and a spontaneous cytokine-independent growth of megakaryocytic colonies isolated from patients with ET. The spontaneous growth is correlated with the allelic load of CALR mutations (44). Comparison of CALR expression in different myeloid cells demonstrated preferential expression of CALR in megakaryocytes is a physiological phenomenon. The expression of CALR in erythroblasts and granulocytes is significantly downregulated from the CD34$^+$ cells, suggesting a crucial role of CALR in the platelet functions.

CALR is expressed on the surface of human platelets, and antibodies against CALR cause platelet activation and induce FcgammaRIIa-independent platelet aggregation. Moreover, surface CALR is associated with collagen receptors on the platelet surface, where it may have a role in the modulation of the platelet-collagen interaction. Furthermore, the interaction of platelet surface CALR with its ligand C1q, which is part of the first component of complement C1, leads to cellular activation followed by release of biological mediators and expression of adhesion molecules, contributing directly or indirectly to the inflammatory process. Stimulation of granulocyte differentiation in HL60 cells induces a rapid decline in CALR mRNA and protein levels. Differentiation also greatly reduced the Ca$^{2+}$ content of HL60 cells, reflecting an ER remodeling as a crucial feature of granulocytic differentiation (45).

**Detection method**

Different from point mutations commonly seen in other altered genes, mutations in CALR are largely insertions and deletions, which are difficult to be detected by conventional Sanger sequencing. That is the reason why CALR mutation was not discovered until recently. There are four methods currently used for the purpose of CALR mutation quantification: Sanger sequencing combined with capillary electrophoresis, HRM analysis, quantitative reverse transcriptase PCR, and next generation sequencing (NGS). The former is the most practical regarding sensitivity and specificity but costly. HRM analysis is based on different
melting curves of the wild type and mutants and provides a rapid and close-system genotyping method (16) and is able to detect even lower allele burden when combined with TA-cloning (16). HRM can classify most of the type 1 and type 2 with occasional misclassification or indeterminate pattern due to similar melting properties of other mutations (16). Therefore, HRM is best used as a screening method. Further test, particularly quantitative reverse transcriptase PCR, is recommended for confirmation. Quantitative reverse transcriptase PCR is able to detected 1% of the mutated DNA in 99% background of wild-type DNA for both types 1 and 2 mutations and is particularly suitable for confirmation of these two most common mutations (16). CALR mutations were originally identified by NGS (12,13,22). The advantage is that it detects the mutations and also quantifies them. Besides, NGS can detect multiple mutations at the same time and also detect copy number alterations (22).

Jones et al. (46) has evaluated the ability of detecting a series of different CALR mutations on four genetic screening methods, including Sanger sequencing, fragment analysis PCR, HRM, and targeted NGS. The limit of detection (LoD) of each assay was tested using serial dilutions made with DNA from CALR positive sample DNA and a cell line, MARIMO, found to carry a heterozygous 61 nucleotide CALR deletion. They found that all methods were capable of detecting each mutation; the most sensitive method was targeted NGS which detected mutations down to 1.25% mutational burden, followed by HRM at 5%, and fragment analysis PCR at 5-10%, and Sanger sequencing at 10-25%.

**Morphological and clinical correlation**

All the previously published data have shown that CALR-mutated PMF patients were younger than their JAK2-mutated counterparts (21), and displayed higher platelet count, lower leukocyte count, lower incidence of spliceosome mutations, and longer survival (21). The hemoglobin levels in PMF with CALR mutations seemed to follow opposite direction to ET and were less likely to display anemia or require transfusion (21). This was likely due to the significantly lower frequency of U2AF1 mutations in CALR-mutated cases (21).

Similar to PMF, ET with CALR mutations were younger and displayed higher platelet count and lower leukocyte count than those with JAK2 mutation As mentioned above, CALR-mutated ET patients had a higher hemoglobin concentration than those with PMF (27). There was a marked female predominance (15,27).

The risk of thrombosis in ET patient with CALR mutation is controversial. Although many studies demonstrated that ET with CALR mutations had higher platelet count than that with JAK2 mutation and a lower incidence of thrombosis (47), it did not retain the association with risk of thrombosis in the multivariable models including 1,150 patients when the risk factors of international prognostic score for the risk of thrombosis in ET are included and is therefore not an independent risk factor. The possible reasons are these patients are younger, have less frequent previous history of thrombosis, and CALR mutation is mutually exclusive with JAK2 mutation (48).

JAK2-mutated ET and CALR-mutated ET have different and distinct biologic and clinical features and may represent two distinct diseases (28). The mutant allele burden was lower in JAK2-mutated ET than in CALR-mutated ET. JAK2-mutated ET has a 29% cumulative risk of progressing to PV, which has significant higher allele burden than ET. However, no polycythemic transformation was observed in CALR-mutated ET. Although transformation to myelofibrosis is associated with the allele burden in both types of ET, there was no significant difference in myelofibrotic transformation between them. Subjects with PMF had a higher CALR mutant allele burden than those with ET. These data are similar to those reported by others in smaller series (28). These studies also suggest an association between CALR mutant allele burden and likelihood of progression of ET to PMF, perhaps of progressive expansion of a heterozygous CALR mutated clone (27).

As discussed above, ASXL1 serves as a modifier for MPNs. In PMF, ‘CALR-/-ASXL1+’ mutation profile is particularly detrimental to survival. CALR-mutated patients fared better than triple-negative cases; the latter also displayed worse leukemia-free survival. Thus, ‘CALR/- ASXL1+’ and ‘triple-negative’ PMF should now be considered as a molecularly high-risk disease (21). However, in multivariable analysis, CALR mutations in PMF had a favorable impact on survival that was independent of both DIPSS+-risk and ASXL1 mutation status which is in contrast to a previous study (21).

Not all CALR mutations follow the same hematologic and prognostic pattern. As discussed previously, CALR mutations can be classified as type 1, type 1-like, type 2, and type 2-like. No significant survival difference existed
between type 1 and type 1-like and between type 2 and type 2-like (37,49). The prognostic advantage of CALR mutations in PMFs might be confined to type 1 or type 1-like mutations, which is consistent with the higher α-helix content of type 2 mutation that is similar to wild-type CALR based on a statistical model. Type 2/type 2-like mutations were associated with higher dynamic international prognostic scoring system (DIPSS)-plus score, EZH2 mutations, leukocyte count >25×10/L, higher circulating blast percentage, and palpable spleen size >10 cm. A comparison of type 1/type 1-like CALR and JAK2 mutations showed the former to be associated with younger age, higher platelet count, higher hemoglobin level, lower leukocyte count, and lower DIPSS-plus score. None of these associations was evident during comparison of type 2-like CALR mutation with JAK2 mutation. Survival was significantly shorter in patients with type 2-like mutation when compared with those with type 1-like CALR mutations, and the difference remained significant when analysis was adjusted for age, ASXL1, or EZH2 mutations. Compared with JAK2-mutated cases, survival was longer in patients with type 1-like but not in those with type 2-like CALR mutations; the difference remained significant adjusted for age, ASXL1 or EZH2 mutations, or DIPSS-plus Score (37). There is no such study has been done for ET.

JAK2-mutated ET patients with concomitant CALR alterations were associated with oldest age (P=0.025), higher thrombotic events after diagnosis (P=0.048), higher major arterial thrombotic events after diagnosis (P=0.022) and more patients being in the high-risk group for thrombohemorrhagic complications (P=0.023) (24). Although PMF with concomitant CALR and JAK2 mutations are present, their significance in clinical presentation or prognosis is unclear due to rarity of the cases.

The correlation between WHO histological criteria and gene mutations has revealed that histological criteria of MPN have a limited diagnostic accuracy due to low sensitivity and patients with JAK2-positive MPN have a heterogeneous histology while CALR-positive ET is associated with megakaryocyte abnormalities and prefibrotic PMF (50).

By exploiting the fact that all CALR mutations generate a novel protein C terminus of a minimum of 17-peptide residue in the variant C terminus of mutated CALR, specific antibodies were developed for the use of immunostaining of bone marrow biopsies. This antibody specifically recognized patients harboring different types of CALR mutation with no staining in healthy controls and JAK2- or MPL-mutated ET and PMF. Therefore, immunostaining could serve as a specific diagnostic tool for molecular categorization of the patients (45).

Since the discovery of CALR mutations in MPNs, the responses of MPNs with CALR mutations to standard therapies have been compared to MPNs with JAK2 mutations. Although CALR mutations offer better survival, no significant difference in response to JAK2 inhibitor was observed between MPNs with CALR mutations and MPNs with JAK2 mutations (51,52). This is in consistent with activated JAK2 signaling pathways in MPNs with CALR mutation. However, the specific characteristics of CALR mutation might offer additional treatment modalities.

Recent studies have shown that calcineurin-NFAT signaling pathway may function as a negative regulator of myeloid/megakaryocytic development at the level of hematopoietic progenitors (53,54) and mutated CALR has been speculated to be able to keep this pathway less active. If this hypothesis is true, it may open the way toward a specific treatment. For instance, it was shown that compounds such as chlorogenic acid can cause calcineurin activation in a calmodulin-dependent manner (41).

Modification of diagnostic criteria

Due to the high prevalence of CALR mutation in JAK2 V617F- and MPL-non-mutated patients, it has been suggested that the presence or absence of CALR mutation be added to the British Committee for Standards in Haematology criteria for the diagnosis of ET and PMF. However, using CALR mutation for risk stratification or proposed International Prognostic Score for ET (IPS-ET) is not recommended. Since these uses have not been tested and the IPS-ET score was tested upon retrospective selected groups of patients who were already receiving treatment (55).

Similarly, a revision of WHO diagnostic criteria for MPNs has been proposed, too (14). The argument is that CALR mutations are frequent in JAK2/MPL-unmutated ET/PMF and thus provide a much needed clonal marker in such cases. In the context of consistent morphology, CALR, as well as JAK2 and MPL mutations, is relatively specific to MPN and should therefore be separated from ‘other’ clonal markers such as abnormal karyotypes. However, CALR mutations do not fully address the molecular gap in JAK2/MPL-unmutated disease or distinguish between ET and early/prefibrotic PMF. As such, their availability does by no
means undercut the necessity of bone marrow morphology as a major diagnostic criterion in both ET and PMF (14).

**Unanswered questions**

There are many questions remained to be answered. There are so many different variants of *CALR* mutations. Only type 1 and type 2 mutations are extensively studies for their correlation with clinical manifestation and prognosis. The clinical significance of other mutations was not carefully studied. Controversies exist regarding the association of *CALR* mutation and risk of thrombosis. Larger scale studies are needed to confirm the beneficial effect of *CALR* mutation in the prognosis of MPNs. Besides, it is too early to conclude that MPNs with *CALR* mutation respond well to current standard care of MPNs based on limited studies.

The understanding of the mechanisms by which *CALR* mutation causes MPNs has remained unknown. More elaborate *in vitro* transfection based experiments are required to fulfill this facet. Animal models with conditional knock-in of *CALR* mutations will be invaluable in clarifying the *in vivo* effect. These studies may provide additional therapeutic targets.

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**Footnote**

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