Review Article

Stem Cell INVESTIGATION

Calreticulin (CALR) mutation in myeloproliferative neoplasms (MPNs)

Wenyi Luo, Zhongxin Yu

Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Contributions: (I) Conception and design: Z Yu; (II) Administrative support: Z Yu; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: W Luo; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Dr. Zhongxin Yu. Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA. Email: zhongxin-yu@ouhsc.edu.

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

Received: 12 July 2015; Accepted: 19 August 2015; Published: 31 August 2015.

doi: 10.3978/j.issn.2306-9759.2015.08.01

View this article at: http://dx.doi.org/10.3978/j.issn.2306-9759.2015.08.01

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 classification2008). 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),

© Stem Cell Investigation. All rights reserved.

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.

© Stem Cell Investigation. All rights reserved.

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 ethnics. 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). Germline 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

© Stem Cell Investigation. All rights reserved.

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 frame-shifting 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 *ASXL* 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 Ca^{2+} 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

© Stem Cell Investigation. All rights reserved.

prototypes for studying the oncogenic mechanisms (37).

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

© Stem Cell Investigation. All rights reserved.

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-plus 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

© Stem Cell Investigation. All rights reserved.

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 presentence 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

© Stem Cell Investigation. All rights reserved.

means undercut the necessity of bone morrow 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 elaborated *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.

Acknowledgements

None.

Footnote

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

References

1. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779-90.
2. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 2006;108:3472-6.
3. Tefferi A, Pardanani A, Lim KH, et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 2009;23:905-11.
4. Carbuccia N, Murati A, Trouplin V, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. Leukemia 2009;23:2183-6.
5. Abdel-Wahab O, Manshouri T, Patel J, et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res 2010;70:447-52.
6. Green A, Beer P. Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. N Engl J Med 2010;362:369-70.
7. Grand FH, Hidalgo-Curtis CE, Ernst T, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 2009;113:6182-92.
8. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia 2010;24:1128-38.
9. Oh ST, Simonds EF, Jones C, et al. Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. Blood 2010;116:988-92.
10. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet 2010;42:722-6.
11. Abdel-Wahab O, Pardanani A, Rampal R, et al. DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. Leukemia 2011;25:1219-20.
12. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 2013;369:2391-405.
13. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 2013;369:2379-90.
14. Tefferi A, Thiele J, Vannucchi AM, et al. An overview on CALR and CSF3R mutations and a proposal for revision of WHO diagnostic criteria for myeloproliferative neoplasms. Leukemia 2014;28:1407-13.
15. Wu Z, Zhang X, Xu X, et al. The mutation profile of JAK2 and CALR in Chinese Han patients with Philadelphia chromosome-negative myeloproliferative neoplasms. J Hematol Oncol 2014;7:48.
16. Chi J, Manoloukos M, Pierides C, et al. Calreticulin mutations in myeloproliferative neoplasms and new methodology for their detection and monitoring. Ann Hematol 2015;94:399-408.
17. Rumi E, Pietra D, Pascutto C, et al. Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis. Blood 2014;124:1062-9.

© Stem Cell Investigation. All rights reserved.

18. Cabagnols X, Defour JP, Ugo V, et al. Differential association of calreticulin type 1 and type 2 mutations with myelofibrosis and essential thrombocytemia: relevance for disease evolution. Leukemia 2015;29:249-52.
19. Park SH, Kim SY, Lee SM, et al. Incidence, clinical features, and prognostic impact of CALR exon 9 mutations in essential thrombocythemia and primary myelofibrosis: an experience of a single tertiary hospital in Korea. Ann Lab Med 2015;35:233-7.
20. Broséus J, Lippert E, Harutyunyan AS, et al. Low rate of calreticulin mutations in refractory anaemia with ring sideroblasts and marked thrombocytosis. Leukemia 2014;28:1374-6.
21. Tefferi A, Lasho TL, Finke CM, et al. CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. Leukemia 2014;28:1472-7.
22. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. Blood 2014;123:2220-8.
23. McGaffin G, Harper K, Stirling D, et al. JAK2 V617F and CALR mutations are not mutually exclusive; findings from retrospective analysis of a small patient cohort. Br J Haematol 2014;167:276-8.
24. Lim KH, Chang YC, Gon-Shen Chen C, et al. Frequent CALR exon 9 alterations in JAK2 V617F-mutated essential thrombocythemia detected by high-resolution melting analysis. Blood Cancer J 2015;5:e295.
25. Malcovati L, Rumi E, Cazzola M. Somatic mutations of calreticulin in myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms. Haematologica 2014;99:1650-2.
26. Nangalia J, Green TR. The evolving genomic landscape of myeloproliferative neoplasms. Hematology Am Soc Hematol Educ Program 2014;2014:287-96.
27. Li N, Yao QM, Gale RP, et al. Frequency and allele burden of CALR mutations in Chinese with essential thrombocythemia and primary myelofibrosis without JAK2(V617F) or MPL mutations. Leuk Res 2015;39:510-4.
28. Rumi E, Pietra D, Ferretti V, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. Blood 2014;123:1544-51.
29. Fu R, Xuan M, Zhou Y, et al. Analysis of calreticulin mutations in Chinese patients with essential thrombocythemia: clinical implications in diagnosis, prognosis and treatment. Leukemia 2014;28:1912-4.
30. Rumi E, Harutyunyan AS, Pietra D, et al. CALR exon 9 mutations are somatically acquired events in familial cases of essential thrombocythemia or primary myelofibrosis. Blood 2014;123:2416-9.
31. Plompen EP, Valk PJ, Chu I, et al. Somatic calreticulin mutations in patients with Budd-Chiari syndrome and portal vein thrombosis. Haematologica 2015;100:e226-8.
32. Nabi MO, Mirabzadeh A, Feizzadeh G, et al. Novel mutations in the calreticulin gene core promoter and coding sequence in schizoaffective disorder. Am J Med Genet B Neuropsychiatr Genet 2010;153B:706-9.
33. Broséus J, Park JH, Carillo S, et al. Presence of calreticulin mutations in JAK2-negative polycythemia vera. Blood 2014;124:3964-6.
34. Lundberg P, Nienhold R, Ambrosetti A, et al. Somatic mutations in calreticulin can be found in pedigrees with familial predisposition to myeloproliferative neoplasms. Blood 2014;123:2744-5.
35. Cabagnols X, Cayuela JM, Vainchenker W. A CALR mutation preceding BCR-ABL1 in an atypical myeloproliferative neoplasm. N Engl J Med 2015;372:688-90.
36. Vorechovsky I, Jones AV, Cross NC. Why do we see JAK2 exon 12 mutations in myeloproliferative neoplasms? Leukemia 2013;27:1930-2.
37. Tefferi A, Guglielmelli P, Lasho TL, et al. CALR and ASXL1 mutations-based molecular prognostication in primary myelofibrosis: an international study of 570 patients. Leukemia 2014;28:1494-500.
38. Prathyuman S, Sellappa S, Joseph S, et al. Enhanced calreticulin expression triggers apoptosis in the MCF-7 cell line. Asian Pac J Cancer Prev 2010;11:1133-6.
39. de Bruyn M, Wiersma VR, Helfrich W, et al. The ever-expanding immunomodulatory role of calreticulin in cancer immunity. Front Oncol 2015;5:35.
40. Mesaeli N, Nakamura K, Zvaritch E, et al. Calreticulin is essential for cardiac development. J Cell Biol. 1999;144:857-68.
41. Shivarov V, Ivanova M, Tiu RV. Mutated calreticulin retains structurally disordered C terminus that cannot bind Ca(2+): some mechanistic and therapeutic implications. Blood Cancer J 2014;4:e185.
42. Rampal R, Al-Shahrour F, Abdel-Wahab O, et al. Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. Blood 2014;123:e123-33.
43. Chao MP, Jaiswal S, Weissman-Tsukamoto R, et al. Calreticulin is the dominant pro-phagocytic signal on

© Stem Cell Investigation. All rights reserved.

multiple human cancers and is counterbalanced by CD47. Sci Transl Med 2010;2:63ra94.
44. Mondet J, Park JH, Menard A, et al. Endogenous megakaryocytic colonies underline association between megakaryocytes and calreticulin mutations in essential thrombocythemia. Haematologica 2015;100:e176-8.
45. Vannucchi AM, Rotunno G, Bartalucci N, et al. Calreticulin mutation-specific immunostaining in myeloproliferative neoplasms: pathogenetic insight and diagnostic value. Leukemia 2014;28:1811-8.
46. Jones AV, Ward D, Lyon M, et al. Evaluation of methods to detect CALR mutations in myeloproliferative neoplasms. Leuk Res 2015;39:82-7.
47. Chen CC, Gau JP, Chou HJ, et al. Frequencies, clinical characteristics, and outcome of somatic CALR mutations in JAK2-unmutated essential thrombocythemia. Ann Hematol 2014;93:2029-36.
48. Finazzi G, Carobbio A, Guglielmelli P, et al. Calreticulin mutation does not modify the IPSET score for predicting the risk of thrombosis among 1150 patients with essential thrombocythemia. Blood 2014;124:2611-2.
49. Tefferi A, Lasho TL, Tischer A, et al. The prognostic advantage of calreticulin mutations in myelofibrosis might be confined to type 1 or type 1-like CALR variants. Blood 2014;124:2465-6.
50. Alvarez-Larrán A, Ancochea A, García M, et al. WHO-histological criteria for myeloproliferative neoplasms: reproducibility, diagnostic accuracy and correlation with gene mutations and clinical outcomes. Br J Haematol 2014;166:911-9.
51. Guglielmelli P, Bartalucci N, Rotunno G, et al. Calreticulin: a new horizon for the testing and treatment of myeloproliferative neoplasms. Expert Rev Hematol 2014;7:423-5.
52. Passamonti F, Caramazza D, Maffioli M. JAK inhibitor in CALR-mutant myelofibrosis. N Engl J Med 2014;370:1168-9.
53. Fric J, Lim CX, Koh EG, et al. Calcineurin/NFAT signalling inhibits myeloid haematopoiesis. EMBO Mol Med 2012;4:269-82.
54. Zaslavsky A, Chou ST, Schadler K, et al. The calcineurin-NFAT pathway negatively regulates megakaryopoiesis. Blood 2013;121:3205-15.
55. Harrison CN, Butt N, Campbell P, et al. Modification of British Committee for Standards in Haematology diagnostic criteria for essential thrombocythaemia. Br J Haematol 2014;167:421-3.

doi: 10.3978/j.issn.2306-9759.2015.08.01
Cite this article as: Luo W, Yu Z. Calreticulin (CALR) mutation in myeloproliferative neoplasms (MPNs). Stem Cell Investig 2015;2:16.

© Stem Cell Investigation. All rights reserved.