The Egyptian Journal of Hospital Medicine (October 2024) Vol. 97, Page 4020-4026

Significance of CAL2 by Immunohistochemistry in Detecting CALR Mutation in Egyptian Patients with BCR- ABL Negative Myeloproliferative Neoplasms

Samar S. Elshazly, Hend A. Nooh, Asmaa Aboubakr, Ghada Nabil Shahin
Clinical pathology Department, National Cancer Institute, Cairo University
Corresponding author: Samar S. Elshazly, **Tel:** +2-010-019-151-97**,**
E-mail: samar.sami@nci.cu.edu.eg**, ORCID:** 0000-0003-3243-4623

ABSTRACT

Background: Essential thrombocythemia (ET) with Primary Myelofibrosis (PMF) and finally Polycythaemia Vera (PV), are considered the three main entities of the Philadelphia-negative MPNs. Mutations in JAKII, together with CALR and Myeloproliferative Leukemia Protein (MPL) genes can be detected in most of cases with Philadelphia-negative MPNs. The discovery of CALR mutations was implicated in better understanding of the molecular mechanisms involved in the pathogenesis of MPNs patients who had no mutations in JAKII or MPL genes. **Patients and methods:** The current study included 56 participants diagnosed with MPNs. Bone marrow trephine biopsy (BMB) was the sample of choice in our study. CALR specific monoclonal antibody (CAL2) by IHC was done for all MPN patients on BMB specimens.

Results: Nearly 82 % of our cases (46/56) were positive for CALR mutation by IHC with sensitivity of 96.4%, and specificity of 88.9%, with positive predictive value of 94.7%, while negative predictive value was 92.3%, and total accuracy of CALR was 93.9%. We found a significant association between JAKII mutation and CALR positive patients (p=0.004) hyper cellular marrow was prevailing in CALR positive patients (p= 0.004). In addition, CALR mutant patients were associated significantly with higher degrees of fibrosis grade III & IV (p= 0.044). However, no significant relation between splenomegaly and CALR positivity was detected in our study.

Conclusion: CAL2 by IHC was a sensitive and specific marker in detecting CALR mutation in patients diagnosed as MPN. Moreover, it was readily available and cost effective than other routine molecular techniques used in detecting such mutation.

Keywords: MPN, CAL2, CALR, ET, PMF.

INTRODUCTION

Other than BCR-ABL1 positive chronic myeloid leukemia (CML), Essential thrombocythemia (ET) with Primary Myelofibrosis (PMF) and finally Polycythaemia Vera (PV), are considered the three main entities of the Philadelphia-negative MPNs, in which there is abnormal proliferation of one or more of the myeloid lineage and integral activation of the signaling pathways involving hematopoiesis with both ET and PV having the implicit to transform into PMF [1]. Mutations in JAKII, together with CALR and Myeloproliferative Leukemia Protein (MPL) genes can be detected in most of cases with Philadelphia-negative MPNs representing the major genetic mutations resulting in the pathogenesis of these diseases [1].

CALR gene, is responsible for encoding a multi-functional CALR protein, which is located in the endoplasmic reticulum, and has a major role in folding of the newly formed proteins and calcium homeostasis [2-4]. Mutations in CALR gene have been found in about 50% up to 80% of cases with MPNs which were negative to JAK2 and MPL gene mutations, and these cases represent about 20% to 35% of all patients with ET and PMF [5].

The discovery of CALR mutations was implicated in better understanding of the molecular mechanisms involved in the pathogenesis of MPNs patients who had no mutations in JAKII or MPL genes. At first, CALR mutation was reported to be mutually exclusive with JAKII and MPL gene mutations, but few studies reported the occurrence of the co-mutation of CALR with either a JAKII or a BCR-ABL1 mutation [6, 7]. The mutational status of these genes is not characteristically associated with a specific type of MPN [1]. Different mutations can occurs in CALR gene with the resulting mutant CALR protein losing the amino acid terminal KDEL sequence in its C-terminal, which probably has impaired Ca^{2+} binding function that eventually result in hyperactivation of the JAK/STAT cascade in megakaryocytes [8]. Mutations of CALR were described by some studies in almost all of JAKII and MPL negative MPNs, with CALR mutations identified in 25–35% of all ET and PMF patient cohorts [9].

The most severe Philadelphia-negative MPN is PMF which is identified by marrow fibrosis together with suppression of all marrow elements and a 5-year survival of less than 50%. Although rare PMF affects young patients, while older adults are the majority of cases and can transform into acute leukemia in > 15% of cases. Being the second most common genetic mutations associated with PMF, CALR mutations are found in 70% of non-JAK2V617F and non-MPL patients and are observed in 20–30% of ET [1]. Unfortunately, patients harboring CALR mutations do not adequately respond to JAK inhibitor therapy and no CALR mutation inhibitor has been developed [10].

Received: 02/07/2024
Accepted: 01/09/2024

Although, the reference methods for detection of these gene mutations are molecular techniques. An immunohistochemistry technique can be used to detect the CALR mutation in formalin-fixed bone marrow biopsy samples and it can be considered as a rapid and inexpensive method for identification of this mutation.

In this study, we assessed the attainment of a new commercially available mutant CALR specific monoclonal antibody (CAL2) applicable to immunohistochemistry in formalin-fixed, decalcified bone marrow biopsy specimens with special emphasis on the diagnostic significance, clinical features associated with those cases and correlating the JAKII mutational status to CAL2 mutation in our cohort.

MATERIAL AND METHODS

Sample collection: The current study included 56 participants newly diagnosed as MPNs. They were recruited from Haematology/oncology outpatient clinics at the National Cancer Institute (NCI), Cairo University.

All of our patients went through detailed history taking, proper clinical examination and laboratory investigations including Complete blood count (CBC), bone marrow aspiration (BMA), bone marrow trephine biopsy (BMB) and molecular techniques for BCR-ABL fusion gene, JAKII, CALR and MPL mutations whenever possible. Initial diagnostic evaluation of MPN depends on close correlation between clinical features, molecular diagnostics, and usually morphologic evaluation of a trephine bone marrow biopsy.

According to the 2016 WHO diagnostic criteria of BCR-ABL negative myeloproliferative neoplasms, distinction between polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) was obtained through integrating peripheral blood findings with molecular data and bone marrow morphologic evaluation findings, as none of these parameters alone provide sufficient diagnostic specificity. Major diagnostic criteria for the diagnosis of PV include elevated haemoglobin concentration and/or hematocrit, accompanied by trilineage hyperplasia (panmyelosis), with pleomorphic mature megakaryocytes in the bone marrow, and JAK2 p.V617F or JAK2 exon 12 mutations. The diagnosis of ET was considered if there was thrombocytosis and proliferation of mature megakaryocytes in the bone marrow that were large in size with hyper lobulated nuclei after the exclusion of reactive thrombocytosis. PMF is characterized by a proliferation of abnormal megakaryocytes and granulocytes in the bone marrow, which is associated in fibrotic stages with a polyclonal increase in fibroblasts that drive secondary reticulin and/or collagen marrow fibrosis, osteosclerosis, and extramedullary haematopoiesis [11].

Aim of the study: In this study, we attempted to assess the diagnostic significance of a mutant CALR specific monoclonal antibody (CAL2) by IHC techniques on bone marrow biopsy specimens and correlating the JAKII mutational status with CAL2 mutation in our patients.

Procedure: Specimens prepared for histological examinations were sectioned from bone marrow biopsy specimens that have been decalcified with 10% formaldehyde, paraffin embedded and multiple sections were prepared and mounting with DPX was done. One of them stained with hematoxylin and eosin (H&E stain) and the other was stained using routine immunohistochemistry staining techniques that allow the visualization of tissue (cell) antigens using CALR antibody (supplied from chongging biopsies Co., Cat number YMA1296) staining used a 1:1,000 dilution for 15 minutes at room temperature, antigen retrieval (EnVision FLEX Target Retrieval Solution; Dako) with a high pH EDTA-based buffer (EnVision FLEX Substrate Buffer; Dako), and a 15-minute Background blocking reagent (EnVision FLEX Peroxidase-Blocking Reagent ; Dako) together with the Autostainer Link-48 and PT Link [12]. For each patient, two slides were examined independently by two haematopathologist consultants using 10x, 40x, x100 magnification of Leica microscope. Examination of the BMB specimen included assessment of cellularity, the percentage and morphology of each cell type moreover number of megakaryocytes and presence of fibrosis were also determined [13].

According to **Nomani *et al.*** [5] the CALR antibody expression was accounted only in the cytoplasm of megakaryocytes as a granular pattern. Expression was graded according to the intensity into dim, moderate and bright where dim was considered as negative expression, while moderate and bright expression were considered positive, (Figures 1 & 2). The same pattern of positivity was observed in the megakaryocytes of ET, PMF and PV. Positive staining was also observed in some granulocytic and erythroid cell lines. However, positivity in non-megakaryocyte was not graded.

Negative control sections were available for 27 non-MPNs patients who presented with Non-Hodgkin Lymphomas (NHL) without infiltrating the bone marrow by lymphoid cells. We followed the same techniques through controls slide preparation as the same steps during CAL2 immunostaining of the MPNs patients, no positive staining in megakaryocytes was detected in the control patients.

Ethical approval: Our protocols followed in this study were approved by Ethics Committee at the National Cancer Institute. Written informed consent was signed by the participants. All the methods were performed in accordance with relevant guidelines and regulations and according to the declaration of Helsinki.

Statistical analysis:

Data were analyzed using SPSS statistical package version 26. Numerical data were summarized as means and standard deviations (SD). While qualitative data were described as frequencies and percentages. Relation between qualitative data was done using the Chi-square test or Fisher exact as appropriate. To compare the two groups of quantitative variables, the Mann-Whitney test was used. Spearman correlation was used to correlate continuous data. The Kappa measure was used to assess the agreement between CALR and JACKII mutation. Receiver operator characteristic (ROC) curves to obtain the optimum cut-off value were used. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Patients' characteristics: The study included 56 participants, the mean age of the participants was 50.2 ± 17.2 years. About two thirds of our patients were males (60.7%). The means of Haemoglobin, total leucocytic count, platelets and LDH were 13.0 ± 3.4gm/dL, 19.5 ± 18.4/cmm^3, 669.2 ± 499.1/µL and 853.9 ± 497 U/L respectively. Splenomegaly was present in 65.4% of the patients. Regarding the bone marrow findings, hypocellular bone marrow was observed in 4 (7.1%) patients, normocellular and hypercellular marrow were found in 18 (32.2%) and 34 (60.7%) patients respectively. Evident fibrosis grade (III & IV) were found in 28 (50%) patients, megakaryocytes were found to be increased in 38 (67.8%) patients (Table1).

Positive cytoplasmic granulations surrounding the nucleus of the megakaryocyte.

Figure (1&2): Megakaryocyte x100

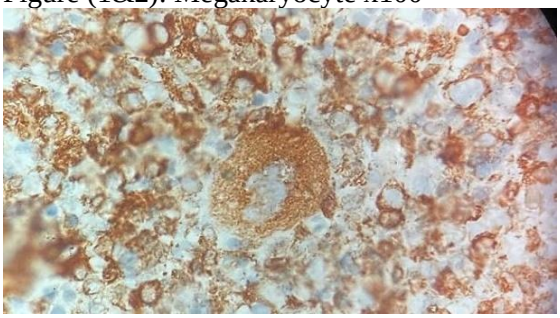

Figure (1)

Absence of cytoplasmic granulations in the cytoplasm of megakaryocyte.

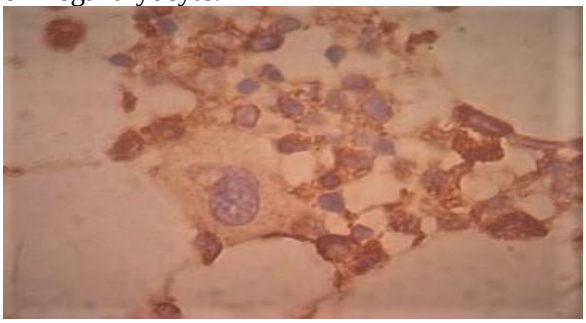

Figure (2)

Table (1): Socio-demographic, clinical, and laboratory data of the participants

Characterises	**Total (n=56)**
Age (years)	50.2±17.2
Sex	
Female	22 (39.3%)
Male	34 (60.7%)
Haemoglobin (mean) (gm/dl)	13.0±3.4
Total leukocyte count (mean) (x10^3)	19.5±4.4
Platelet Count (mean)	669.2±99.1
LDH (mean)	853.9±97.0
Splenomegaly (n=52)	34 (65.4%)
BMB cellularity	
Hypocellular	4 (7.1%)
Normocellular	18 (32.2%)
Hypercellular	34 (60.7%)
BMB fibrosis	
0	14 (25%)
I	14 (25%)
II	12 (21.4%)
III	14 (25%)
IV	2 (3.6%)
BMB MEGs	
Reduced	6 (10.7%)
Normal	12 (21.4%)
Increased	38 (67.8%)

CALR AND JAKII MUTATION STATUS

Out of 56 participants CALR antibody by IHC was positive in 46/56 (82.1%) patient's cohort. JAKII mutation was done for only 44/56 patients in our cohort and it was positive in 20/44(45.5%) patients. Nearly half of the patients (46.4%) were diagnosed with ET, 14 (25%) were MF and 16 (28.6%) were PV.

The proportion of CALR positive patients in each subtype of BCR/ABL negative MPNs were as follow: ET, PV and MF were 21/26 (80.7%), 12/16(75%) and 14/14 (100%), respectively were positive for CALR mutation by IHC, 44 cases underwent JAKII mutation analysis by molecular techniques and about 56% were positive for both JAKII and CALR (table 2).

Table (2): CALR and JAKII mutation status

CALR Positivity	
Negative	10 (17.9%)
Positive	46 (82.1%)
JAKII by molecular (n=44)	
Negative	24 (54.5%)
Positive	20 (45.5%)
Diagnosis	
MF	14 (25%)
PV	16 (28.6%)
ET	26 (46.4%)
CALR positive, BCR/ABL negative MPNs	
ET	21/26 (80.7%)
PV	12/16(75%)
MF	14/14 (100%)

Assessment of the diagnostic accuracy of CALR in MPN patients:

The best cut-off point was 65, with the area under the curve was 0.985, 95% Confidence interval (0.967-1.000), and the CALR had a sensitivity of 96.4%, specificity of 88.9%, positive predictive value of 94.7%, while negative predictive value was 92.3% and total accuracy of CALR was 93.9% (Table 3 & Figure 3).

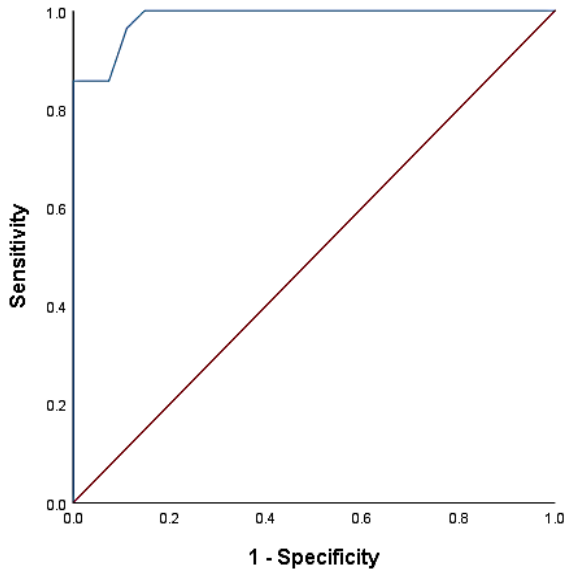

Figure (3): The diagnostic accuracy of CALR in MPN patients

Table (3): The diagnostic accuracy of CALR

Positivity	
Cut off point	≥ 65
Sensitivity	96.4%
Specificity	88.9%
Positive predictive value	94.7%
Negative predictive value	92.3%
Total Accuracy	93.9%
The area under the curve (95% CI)	0.985 (.967-1.000)

Correlation between the expression of CALR mutation and laboratory data:

There was a significant weak negative correlation between CALR positive patients and haemoglobin (r= -0.284, p= 0.034). However no significant correlation between TLC, platelet count, LDH, number of megakaryocytes, and CALR positive patients, (Table 4).

Table (4): Correlation between positive CALR and patient's laboratory data

	CALR mutants	
	Correlation coefficient (r)	**p-value**
Haemoglobin	-0.284	0.034
Total leukocyte count	0.074	0.589
Platelet Count	-0.041	0.766
LDH	-0.401	0.155
No. of Megakaryocytes	0.070	0.608

Correlation between both CALR expression and JAKII mutation and Splenomegaly, BMB cellularity and Fibrosis: Out of our 56 patients only 44/56 underwent JAKII mutation analysis; CALR and JAKII mutations were significantly concordant in 28/44 (63.6%) patients, where 20 patients were both CALR & JAKII mutant positive and 8 patients were CALR and JAKII mutant negative, that's why a fair agreement between JAKII mutation and CALR was observed in our study, Kappa= 0.314 (p= 0.004). Hyper cellular B **Correlation** M was a dominating finding in CALR positive patients (69.6% vs. 20%, p= 0.004). In addition, CLAR mutant patients were associated significantly with higher degrees of fibrosis grade III & IV (30.4% vs. 0%, p= 0.044). No significant relation between splenomegaly and CALR positivity was detected in our study as shown in table (5).

Table (5): Correlation between CALR expression and JAKII mutants, Splenomegaly, BMB cellularity and degree of fibrosis

	CALR mutants		
	Positive (n=46)	**Negative (n=10)**	**p-value**
JAKII mutants (n-44)			
Positive	20 (55.6%)	0 (0.0%)	0.004
Negative	16 (44.4%)	8 (100.0%)	
Splenomegaly (n=52)			
No	14 (31.8%)	4 (50.0%)	0.320
Yes	30 (68.2%)	4 (50.0%)	
BMB cellularity			
Normocellular & hypocellular	14 (30.4%)	8 (80.0%)	0.004
Hpercellualr	32 (69.6%)	2 (20.0%)	
Fibrosis			
0&I&II	32 (69.6%)	10 (100.0%)	0.044
III&IV	14 (30.4%)	0 (0.0%)	

DISCUSSION

CALR gene is allocated on chromosome 19p13.2, it consists of 9 exons, and is about 4.2 kb. Calreticulin maintains normal Ca^{2+} level, the removal of improperly folded proteins, cellular adhesion, immunologic responses to tumors and involved in cell signaling [14-16].

CALR mutation is considered a gain of function mutation, which induces MPL pathway activation. Interacting with MPL, mutant CALR activates signaling molecules that also activates JAKII and further cytokine independent proliferation. The exact molecular mechanism by which CALR consecutively activates MPL is still not clear. Moreover testing of CALR has been established as a diagnostic criterion on the revised WHO criteria for ET and PMF [17]. The well-known driver gene mutations in MPNs neoplasms are JAKII, CALR and MPL mutations. The CALR as well as JAKII mutations are considered potential risk factors for thrombosis, marrow fibrosis in addition to disease progression [18-20].

In the present study, we were testing the diagnostic accuracy of CALR mutation by using CAL2 IHC and trying to correlate this mutation with JAKII mutation and other laboratory parameters in BCR-ABL negative MPN patients. We found that our results are in agreement with reports of **Stein *et al.*** [21] regarding the sensitivity, specificity and total accuracy of CAL2 as a marker for detection of CALR mutation by IHC. **Stein *et al.*** [21] was the first to describe CALR mutation detection by CAL2 immunohistochemistry in MPNs, and we like him consider this method as a highly sensitive and specific method for detection of the CALR mutation.

Other studies were done to assess and compare CAL2 mutation status done with immunohistochemistry and other molecular techniques, which revealed, like us, complete concordance between the results of the two different techniques [22, 23].

We found in line with other studies, the positivity of CAL2 staining was clearly evident in the cytoplasm of megakaryocytes of the studied patients' cohort [5, 21, 24]. The possible explanation that the positivity of CALR staining is confined to the cytoplasm of megakaryocytes could be the accumulation and overexpression of wild type CALR protein occur in megakaryocytes more than any other cell types [25]. Moreover, we are in agreement with others [22, 23] that CAL2 protein positive staining was observed in non-megakaryocytic cell lines mainly granulocytic and erythroid cells, which can be attributed to the fact that the emergence of CALR mutation appear early in multipotent progenitors, which differentiate into myeloid and erythroid progenitors [3].

We found that the occurrence of CALR mutation in ET and MF patients regardless of JAKII mutation status are in close agreement with other studies [2, 3] where they found that the average incidence of CALR mutation in ET and MF was 70% and 90% respectively, which is quite similar to our results, however those results are in contrast to **Kang *et al.*** [26] who found that the incidence of the mutation was lower than in our cohort, which could be attributed to racial backgrounds. It's well-known that mutations in CALR, MPL or JAKII are mutually exclusive and the occurrence of a mutation in one gene does not necessitate a mutations in other driver genes in Ph- MPNS [27, 28].

In our study, we found that CALR and JAKII are in accordance with each other whether double positive mutants or double negative, these findings are found to be in agreement with recent reports and case studies, which declares the possible association between CALR mutation and JAKII mutations and could explain the interrelating clinical and laboratory findings in Ph- MPNs [29-33].

Regarding the relation of CALR mutation and patients' laboratory parameters, we found like other studies that mutant CALR is associated with lower hemoglobin levels. However, these studies compared CALR positive patients and JAKII positive patients, which we couldn't conduct due to small sample size [2, 3].

In agreement with previous reports, we found that the CALR mutation was associated with increased marrow fibrosis. However we couldn't find statistical association with CALR mutations and splenomegaly or LDH [18, 34, 35], which could be explained with different patients sociodemographic conditions, or smaller sample size.

In agreement with **Heba *et al.*** we could strongly account on CAL2 monoclonal antibody in accurately revealing mutant CALR in BMB. We also showed that the immune reactivity of CAL2 was entirely restricted to the presence of CALR mutations, which were found only in MPN biopsies, but not in lymphomas biopsies of the control specimens [23].

CONCLUSION

CAL2 by IHC was a sensitive and specific marker in detecting CALR mutation in patients diagnosed as MPN. Moreover it is readily available and cost effective than the routine molecular techniques used in detecting such mutation. There was also a significantly higher proportion of JAKII mutation positive cases and positive cases by CALR (55.6% versus 0%, P-value = 0.004) than in negative JAKII mutation.

No funding.
No conflict of interest.

REFERENCES

1. **Tvorogov D, Thompson-Peach C, Foßelteder J *et al*. (2022):** Targeting human CALR-mutated MPN progenitors with a neoepitope-directed monoclonal antibody. EMBO reports., 23: e52904; https://doi.org/10.15252/embr.202152904.
2. **Klampfl T, Gisslinger H, Harutyunyan A *et al*. (2013):** Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med., 369: 2379–90; PMID: 24325356 DOI: 10.1056/NEJMoa1311347
3. **Nangalia J, Massie C, Baxter E *et al*. (2013):** Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med., 369: 2391–405. PMID: 24325359,PMCID: PMC3966280 DOI: 10.1056/NEJMoa1312542
4. **Bonzheim I, Mankel B, Klapthor P *et al*. (2015):** CALR-mutated essential thrombocythemia evolving to chronic myeloid leukemia with coexistent CALR mutation and BCR-ABL1 translocation. Blood. DOI: 10.1182/blood-2014-12-616847125: 2309-2311.
5. **Nomani L, Bodo J, Zhao x *et al*. (2016):** CAL2 Immunohistochemical Staining Accurately Identifies CALR Mutations in Myeloproliferative Neoplasms. Am J Clin Pathol., 146: 431-438. DOI: 10.1093/AJCP/AQW135.
6. **Cabagnols X, Cayuela J, Vainchenker W (2015):** CALR mutation preceding BCR-ABL1 in an atypical myeloproliferative neoplasm. N Engl J Med., 372: 688-690 DOI: 10.1056/NEJMc1413718.
7. **Wang W, Groenendyk J, Michalak M (2012):** Calreticulin signaling in health and disease. Int J Biochem Cell Biol., 44: 842-846 .DOI: 10.1016/j.biocel.2012.02.009
8. **Chao M, Majeti R, Weissman I (2012):** Programmed cell removal: a new obstacle in the road to developing cancer. Nat Rev Cancer, 12: 58-67 . DOI: 10.1038/nrc3171
9. **Loghavi S, Pemmaraju N, Kanagal-Shamanna R *et al*. (2015):** Insights from response to tyrosine kinase inhibitor therapy in a rare myeloproliferative neoplasm with CALR mutation and BCR-ABL1. Blood, 125: 3360-3363 . DOI: 10.1182/blood-2015-03-632893
10. **Ross D, Babon J, Tvorogov D, Thomas D (2021):** Persistence of myelofibrosis treated with ruxolitinib: biology and clinical implications. Haematologica, 106: 1244–1253 . DOI: 10.3324/haematol.2020.262691.
11. **Khoury J, Solary E, Abla O, Akkari Y, Alaggio R *et al*. (2022):** The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia, https://doi.org/10.1038/s41375-022-01613-1.
12. **Lee S, Erber W, Porwit A *et al.* (2008):** ICSH guidelines for standerization of bone marrow spicemen and reports. Int J Lab Hematol., 30: 349-364 . DOI: 10.1111/j.1751-553X.2008.01100.x
13. **Prophet E, Mills B, Arrinqton J *et al.* (1992):** Laboratory Methods in Histotechnology (Armed Forces Institute of Phatology). American Registry of Pathology, Washington.
14. **Eggleton P and Michalak M (2013):** Calreticulin for better or for worse, in sickness and in health, until death do us part. Cell Calcium, 54: 126-31. doi: 10.1016/j.ceca.2013.05.006.
15. **Nakamura K, Robertson M, Liu G *et al.* (2001):** Complete heart block and sudden death in mice overexpressing calreticulin. J Clin Invest., 107: 1245- 53. doi: 10.1172/JCI12412.
16. **Raghavan M, Wijeyesakere S, Peters L, Del Cid N (2013):** Calreticulin in the immune system: ins and outs. Trends Immunol., 34: 13-21. doi: 10.1016/j.it.2012.08.002.
17. **Swerdlow S, Campo E, Harris N *et al.* (2017):** WHO classification of tumours of haematopoietic and lymphoid tissues, Revised 4th Edition, Volume 2. Lyon, France: IARC Press, Pp: 39-53
18. **Gango A, Mozes R, Boha Z *et al.* (2018):** Quantitative assessment of JAK2 V617F and CALR mutations in Philadelphia negative myeloproliferative neoplasms. Leuk Res., 65: 42–8. https://doi.org/10.1016/j.leukres.2017.12.005
19. **Borowczyk M, Wojtaszewska M, Lewandowski K *et al.* (2015):** The JAK2 V617F mutational status and allele burden – authors' reply. Thromb Res., 136: 691–2. DOI: 10.1016/j.thromres.2015.07.006
20. **Li N, Yao Q, Gale R *et al.* (2015):** Frequency and allele burden of CALR mutations in Chinese with essential thrombocythemia and primary myelofibrosis without JAK2(V617F) or MPL mutations. Leuk Res., 39: 510–4. DOI: 10.1016/j.leukres.2015.02.006.
21. **Stein H, Bob R, Durkop H *et al.* (2016):** A new monoclonal antibody (CAL2) detects CALRETICULIN mutations in formalin-fixed and paraffin-embedded bone marrow biopsies. Leukemia, 30: 131–5. doi: 10.1038/leu.2015.192.
22. **Mozes R, Ángo A, Sulak A *et al.* (2019):** Calreticulin mutation specific CAL2 immunohistochemistry accurately identifies rare calreticulin mutations in myeloproliferative neoplasms. Pathology, 51 (3): 301–307. DOI: 10.1016/j.pathol. 2018.11.007.

23. **Ali H, Puccio I, Akarca A *et al.* (2020):** Megakaryocytes, erythropoietic and granulopoietic cells express CAL2 antibody in myeloproliferative neoplasms carrying CALR gene mutations. Int. J. Exp. Path., 00: 1–6. DOI: 10.1111/iep.12375
24. **Andrici J, Farzin M, Clarkson A *et al.* (2016):** Mutation specific immunohistochemistry is highly specific for the presence of calreticulin mutations in myeloproliferative neoplasms. Pathology, 48: 319–24. DOI: 10.1016/j.pathol.2016.03.002.
25. **Salati S, Prudente Z, Genovese E *et al.* (2017):** Calreticulin affects hemato-poietic stem/progenitor cell fate by impacting erythroid and megakar-yocytic differentiation. Stem Cells Dev., 27: 225–36. DOI: 10.1089/scd.2017.0137.
26. **Kang M, Choi1 H , Lee J , Choi Y , Choi H (2016):** Coexistence of JAK2 and CALR mutations and their clinical implications in patients with essential thrombocythemia. Oncotarget, 7 (35).2016, DOI: 10.18632/oncotarget.10958.
27. **Mansier O, Migeon M, Etienne G, Bidet A, Lippert E (2016):** JAK2V617F and CALR double mutations are more frequently encountered in patients with low JAK2V617F allelic burdens. Leuk Lymphoma, 57: 1949- 1951. DOI: 10.3109/10428194.2015.1116122.
28. **Thompson E, Nguyen T, Kankanige Y *et al.* (2021):** Clonal independence of JAK2 and CALR or MPL mutations in comutated myeloproliferative neoplasms demonstrated by single cell DNA sequencing. Haematologica, 106: 313-315. DOI: 10.3324/haematol.2020.260448.
29. **Schueller C, Majoros A,** Novartis **H, Kralovics R (2022):** Co-expression of mutated Jak2 and Calr enhances myeloproliferative phenotype in mice without loss of stem cell fitness. AJH., 97, (11):E396-E399 . DOI: 10.1002/ajh.26688
30. **Mc Gaffin G, Harper K, Stirling D, McLintock L (2014):** JAK2 V617F and CALR mutations are not mutually exclusive; findings from retrospective analysis of a small patient cohort. Br. J. Haematol., 167 (2) :276–278. doi: 10.1111/bjh.12969
31. **Rashid M, Ahmed R, Ahmed S *et al.* (2016):** Coexisting JAK2V617F and CALR Exon 9 Mutation in Essential Thrombocythemia. Indian J. Hematol. Blood Transfus., 32: 112–116. doi: 10.1007/s12288-016-0658-y.
32. **Tefferi A, Lasho TL, Finke C *et al.* (2014):** CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: Clinical, cytogenetic and molecular comparisons. Leukemia, 28: 1472–1477. doi: 10.1038/leu.2014.3.
33. **Nishimura M, Nagaharu K, Ikejiri M *et al.* (2021):** Acquisition of JAK2 V617F to CALR-mutated clones accelerates disease progression and might enhance growth capacity. *Br J Haematol*., 194: e89- e92. DOI: 10.1111/bjh.17571.
34. **Andrikovics H, Krahling T, Balassa K *et al.* (2014):** Distinct clinical characteristics of myeloproliferative neoplasms with calreticulin mutations. Haematologica, 99: 1184–90. DOI: 10.3324/haematol.2014. 107482.
35. **Oh Y, Song I, Kim J, Kwon G, Koo S, Kim S (2018):** Pyrosequencing-based quantitative measurement of CALR mutation allele burdens and their clinical implications in patients with myeloproliferative neoplasms. Clin Chim Acta, 483: 183–91. DOI: 10.1016/j.cca.2018.05.001.