



Calreticulin Exon 9 Mutations in Myeloproliferative Neoplasms

Jung-Sook Ha, M.D.¹ and Yu-Kyung Kim, M.D.²

Department of Laboratory Medicine¹, Keimyung University School of Medicine, Daegu; Department of Laboratory Medicine², Yeungnam University College of Medicine, Daegu, Korea

Background: Calreticulin (*CALR*) mutations were recently discovered in patients with myeloproliferative neoplasms (MPNs). We studied the frequency and type of *CALR* mutations and their hematological characteristics.

Methods: A total of 168 MPN patients (36 polycythemia vera [PV], 114 essential thrombocythemia [ET], and 18 primary myelofibrosis [PMF] cases) were included in the study. *CALR* mutation was analyzed by the direct sequencing method.

Results: *CALR* mutations were detected in 21.9% of ET and 16.7% of PMF patients, which accounted for 58.5% and 33.3% of ET and PMF patients without Janus kinase 2 (*JAK2*) or myeloproliferative leukemia virus oncogenes (*MPL*) mutations, respectively. A total of five types of mutation were detected, among which, L367fs*46 (53.6%) and K385fs*47 (35.7%) were found to be the most common. ET patients with *CALR* mutation had lower leukocyte counts and ages compared with *JAK2*-mutated ET patients.

Conclusion: Genotyping for *CALR* could be a useful diagnostic tool for *JAK2*-or *MPL*-negative ET or PMF patients. *CALR* mutation may be a distinct disease group, with different hematological characteristics than that of *JAK2*-positive patients.

Key Words: Calreticulin, *JAK2*, *MPL*, Myeloproliferative neoplasm

Received: May 20, 2014
Revision received: July 7, 2014
Accepted: November 11, 2014

Corresponding author: Jung-Sook Ha
Department of Laboratory Medicine,
Keimyung University School of Medicine,
56 Dalseong-ro, Jung-gu, Daegu 700-712,
Korea
Tel: +82-53-250-7266
Fax: +82-53-250-7275
E-mail: kkskmom@dsmc.or.kr

© The Korean Society for Laboratory Medicine

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

The recurrent Janus kinase 2 (*JAK2*) V617F mutation has been an important molecular marker for myeloproliferative neoplasms (MPNs) since its discovery in 2005 [1]. Polycythemia vera (PV) is almost invariably associated with *JAK2* mutations (*JAK2* V617F and *JAK2* exon 12 mutation), whereas only 50-60% of essential thrombocythemia (ET) and primary myelofibrosis (PMF) cases are associated with the *JAK2* V617F mutation. Among the 40% of patients with ET and PMF who lack the *JAK2* V617F mutation, 3-5% carry mutations at codon 515 of the gene encoding the thrombopoietin receptor, myeloproliferative leukemia virus oncogene (*MPL*) [2]. These *JAK2* mutations and *MPL* W515 mutations are gain-of-function mutations that induce overactivation of the signal transducer and transcription (STAT)-

signaling activator by autophosphorylation of *JAK2*. The close associations between *JAK2* mutations and MPNs led to the revision of the WHO diagnostic criteria in 2008; these mutations are included in the new major diagnostic criteria for the three classic MPNs.

Other studies reported mutations such as *EZH2*, *ASXL1*, *TET2*, *DNMT3A*, and *IDH1/2* in MPN patients. However, these mutations exist in a small fraction of patients or are detected in other myeloid disorders and not only in MPN. Therefore, as these mutations are not exclusive to *JAK2* V617F, they cannot serve as suitable diagnostic markers for classic MPNs [3].

Recently, two independent research groups reported a recurrent calreticulin (*CALR*) somatic mutation in MPN patients through an exome sequencing approach [4, 5]. Most of these mutations are located in exon 9 and are of insertion/deletion

types. Although these mutations are variable, the *CALR* mutations collectively result in the development of one base pair frameshift and one mutant protein with a novel C terminus. Albeit some rare exceptions, *CALR* mutations are mutually exclusive to *JAK2* mutation or *MPL* mutation and occur in ET and PMF patients, but not in PV patients. Therefore, *CALR* mutation was suggested as a powerful diagnostic tool for patients with ET or PMF that are negative for *JAK2/MPL* mutations.

In this study, we investigated the usefulness of *CALR* mutations as a diagnostic marker in MPN patients. For this purpose, the frequency and type of *CALR* mutations as well as the hematological characteristics were analyzed.

METHODS

The current study was approved by our institutional review board (IRB14-035), and informed consent was obtained from all patients involved. DNA samples that were collected from MPN patients at the time of diagnosis or first referral and then stored were used for this study. The DNA samples were collected from the bone marrow or peripheral blood. A total of 168 MPN patients (85 men, 83 women; mean age 62.8 yr) were included in this study. The patients consist of 36 PV, 114 ET, and 18 PMF. The clinical and laboratory data were reviewed from medical records.

For analysis of *CALR* mutations, the genomic DNA was extracted from cells by using the QIAamp DNA Mini Kit (QIAGEN GmbH; Hilden, Germany). Oligonucleotide primers targeting exon 9 of *CALR* were used to amplify a 483-bp product. The primers were *CALR* (forward) 5'-CATACGCTGAGGAGTTTGGC-3' and (reverse) 5'-GAGTGGAGGAGGGGAACAAA-3'. DNA was amplified by using the Smart Tag Pre-mix (Solgent Co., Daejeon, Korea). Briefly, 200 ng of DNA template and 1 µL each of forward and reverse primers were added to the PCR premix (20 µL final volume). The amplification parameters included an initial denaturation step at 94°C for 30 sec, annealing at 63°C for

30 sec, and extension at 72°C for 60 sec. The quality and sizes of the PCR products were assessed by electrophoresis on 1.5% agarose gel. Products were purified and bidirectionally sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the ABI 3730XL DNA Analyzer (Applied Biosystems).

The *JAK2* V617F and *MPL* mutations were analyzed by using the *JAK2* V617F Kit (Biosewoom, Seoul, Korea) and the Real-Q *MPL* W515L/K Screening Kit (Biosewoom), respectively. *JAK2* exon 12 mutation was analyzed by the direct sequencing method using primers forward 5'-CATACGCTGAGGAGTTTGGC-3'; *JAK2* reverse 5'-GAGTGGAGGAGGGGAACAAA-3'.

The differences in the distribution of continuous variables between the categories were analyzed by either the Mann-Whitney test (for nonparametric analysis) or the independent t-test (for parametric analysis). $P < 0.05$ was considered statistically significant. The SPSS 20.0 (SPSS Inc., Chicago, IL, USA) statistical program was used for all calculations.

RESULTS

From the 168 patients tested, *CALR* mutations were detected in 28 patients (16.7% of all MPNs). The *CALR* mutational frequencies in each subtype were 0% for PV, 21.9% (25/114) for ET, and 16.7% (3/18) for PMF. The *JAK2* mutational frequencies were 86.1% (31/36) for PV, 59.6% (68/114) for ET, and 50.0% (9/18) for PMF (Table 1). *JAK2* exon12 mutations were not detected in any patient. *MPL* mutations were detected only in the ET patients (4.4%; 5/114). For patients with no *JAK2* or *MPL* mutation, the *CALR* mutational frequency was 58.5% (24/41) for ET and 33.3% (3/9) for PMF. One ET patient had both *CALR* and *JAK2* V617F mutations. The incidence of triple-negative (negative for *JAK2/MPL/CALR*) patients was 13.9% (5/36) for PV, 14.0% (16/114) for ET, and 33.3% (6/18) for PMF.

A total of five types of *CALR* mutations were detected in this study (Fig. 1). Among them, L367fs*46 was the most common

Table 1. The frequency of *JAK2* V617F, *MPL* W515, and *CALR* mutations

	PV (n = 36)	ET (n = 114)*	PMF (n = 18)	All patients (n = 168)
<i>JAK2</i> V617F, n (%)	31 (86.1)	68 (59.6)	9 (50.0)	108 (64.3)
<i>MPL</i> W515, n (%)	0 (0.0)	5 (4.4)	0 (0.0)	5 (3.0)
<i>CALR</i> mutant, n (% of total, % of <i>JAK2/MPL</i> -negative mutants)	0 (0.0, 0.0)	25 (21.9, 58.5)	3 (16.7, 33.3)	28 (16.7, 50.9)
Triple-negative†, n (%)	5 (13.9)	16 (14.0)	6 (33.3)	27 (16.1)

*One ET patient had both *CALR* and *JAK2* V617F mutations; †Negative for *JAK2* (V617F and exon 12 mutation), *MPL* W515, and *CALR* mutation. Abbreviations: *JAK2*, Janus kinase 2; *MPL*, myeloproliferative leukemia virus oncogene; *CALR*, calreticulin; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis.

type (53.6%; 15/28), followed by K385fs*47 (35.7%; 10/28), E364fs*55 (3.6%; 1/28), and E369fs*46 (3.6%; 1/28). One ET patient harbored another type of K385fs*47 which was combined with an A deletion and a TTTGTC insertion (1154_delins TTTGTC), which was different from common K385fs*47 having a TTGTC insertion (Table 2).

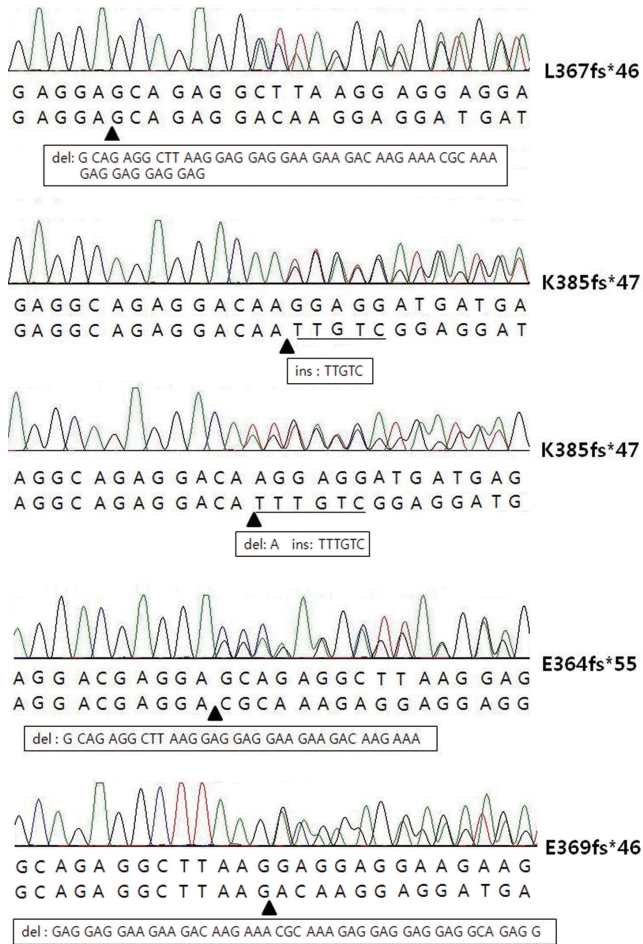


Fig. 1. The five types of calreticulin mutations identified in this study. The arrowheads indicate the points where insertion or deletion occurred. Square boxes represent nucleotide sequences that were deleted or inserted.

Table 2. Type of *CALR* mutations identified in this study

Nucleotide changes	Amino acid changes	Modified C-terminal amino acids	N (%)
Wild type	KQDEEQRLKEEEEDKKRKEEEEAEDKEDDEKDEDEEEDKEEEDVPGQAKDEL	
c.1092_1143del	L367fs*46	TRRMMRTKMRMRMRRTTRRKMRKMSPARPRTSCREACLQGWTEA	15 (53.6)
c.1154_1155insTTGTC	K385fs*47	NCRRMMRTKMRMRMRRTTRRKMRKMSPARPRTSCREACLQGWTEA	10 (35.7)
c.1154delinsTTTGTCT	K385fs*47	ICRRMMRTKMRMRMRRTTRRKMRKMSPARPRTSCREACLQGWTEA	1 (3.4)
c.1092_1125del	E364fs*55	EAKRRRRQRTRRMMRTKMRMRMRRTTRRKMRKMSPARPRTSCREACLQGWTEA	1 (3.4)
c.1105_1150del	E369fs*46	TRRMMRTKMRMRMRRTTRRKMRKMSPARPRTSCREACLQGWTEA	1 (3.4)

On comparing the hematological parameters between *CALR* and *JAK2* mutants, ET patients with *CALR* mutations were younger ($P=0.025$) and had a lower leukocyte count ($P<0.001$). Hemoglobin and platelet counts were not statistically different between the two groups. In PMF patients, no significant differences were noted in the age and the hemoglobin, leukocyte, and platelet counts (Table 3).

DISCUSSION

CALR is a highly conserved, multifunctional endoplasmic reticulum (ER) protein. Functionally, *CALR* plays an integral role in calcium homeostasis and protein folding inside the ER [6]. In addition, outside the ER, *CALR* regulates integrin-mediated cell adhesion, gene nuclear transport, programmed cell removal, and immunogenic cell death. The C-domain of this protein is involved in calcium regulation and contains a KDEL sequence, which is responsible for preventing the secretion of protein from the ER. *CALR* encoding *CALR* protein is located on chromosome 19p13.2, contains 9 exons, and spans a 4.2-kb region [7].

Recently, *CALR* mutations were reported in ET and PMF patients with non-mutated *JAK2/MPL* [4, 5]. All *CALR* mutations reported in MPNs were located on exon 9 and were of the insertion/deletion type. Although the mutations are variable, all mutations cause a frameshift to a unique alternative reading frame. This new reading frame results in the loss of most of the acidic domain, including the KDEL signal, and creates a novel C-terminus protein comprising a minimum of 36 amino acids [4]. This novel sequence contains several positively charged amino acids, whereas the wild-type sequence is mostly negatively charged. Moreover, the exact pathological role of the *CALR* mutation has not yet been discovered. However, a mutated novel C terminus is suggested to disrupt the ER-signaling peptide and influence the *CALR* subcellular localization, stability, function, or any combination of these [4].

Table 3. Hematological characteristics of *CALR* mutant and *JAK2 V617F* mutant in ET and PMF patients

Variables	<i>CALR</i> mutant	<i>JAK2</i> mutant	<i>P</i>
ET			
Number	25	68	
Men:Women	11:14	34:34	
Age (years), median (range)	58 (18-84)	68 (24-86)	0.025
Hemoglobin, g/dL, median (range)	12.9 (8.6-16.5)	13.7 (5.2-17.6)	0.088
Leukocyte, ×10 ⁹ /L, median (range)	9.9 (1.6-23.6)	14.0 (5.2-128.3)	<0.001
Platelet, ×10 ⁹ /L, median (range)	1,150.0 (370.0-2,507.0)	1,068.5 (579.0-1,917.0)	0.121
PMF			
Number	3	9	
Men:Women	1:2	5:4	
Age (years), median (range)	62 (53-79)	72 (49-79)	0.864
Hemoglobin, g/dL, median (range)	7.5 (6.0-8.1)	7.6 (3.8-9.9)	0.864
Leukocyte, ×10 ⁹ /L, median (range)	9.0 (3.7-34.8)	6.7 (3.1-33.5)	0.727
Platelet, ×10 ⁹ /L, median (range)	238.0 (132.0-265.0)	79.0 (9.0-335)	0.064

Abbreviations: see Table 1.

Table 4. The frequencies of *CALR* mutation reported by previous studies

	Subject	Detection methods	Frequency of <i>CALR</i> mutation (%; frequencies for <i>JAK2</i> - or <i>MPL</i> -negative subjects)	Both <i>JAK2</i> (+)/ <i>CALR</i> (+)
Klampfl <i>et al.</i> [4]	1,107 MPN	Whole-genome sequencing and capillary fragment analysis	ET (67), PMF (88)	
Nangalia <i>et al.</i> [5]	151 MPN	Exome sequencing	ET (82), PMF (80)	
	669 MPN	Sanger sequencing	ET (71), PMF (56)	
Tefferi <i>et al.</i> [7]	254 PMF	Sanger sequencing	PMF (74)	1 PMF
Rotunno <i>et al.</i> [9]	576 ET	Sanger sequencing	ET (48.9)	
Rumi <i>et al.</i> [10]	717 ET	Sanger sequencing	ET (63.1)	
Li <i>et al.</i> [11]	357 PMF	Sanger sequencing	PMF (43)	
This study	168 MPN	Sanger sequencing	ET (58.5), PMF (33.3)	1 ET

Abbreviations: MPN, myeloproliferative neoplasms; *JAK2*, *MPL*, *CALR*, ET, PMF; see Table 1.

In MPNs, *CALR* mutations have been found only with ET and PMF, but not with PV. Despite few reports on cases harboring both *JAK2* and *CALR* mutations [7, 8], in this study, almost all *CALR* mutations were found in ET/PMF without any *JAK2/MPL* mutations. In prior reports, the frequency of *CALR* mutation in *JAK2/MPL* negative cases was 48.9-82% for ET and 43-88% for PMF [4, 5, 7, 9-11]. This diverse frequency range can be attributed to the difference in the method of patient selection, sample size, and sensitivity of the detection methods. In studies using whole-genome sequencing method or capillary fragment analysis, the frequency of *CALR* was visibly greater than that of *CALR* in other studies that used direct sequencing methods (Table 4). In our study, the direct sequencing method demon-

strated *CALR* mutation in 58.5% and 33.3% of the *JAK2/MPL*-negative ET and PMF patients, respectively. Its frequency in ET was similar to that reported in prior studies using direct sequencing methods. However, for PMF patients, the frequency of *CALR* (33.3%) was markedly low, probably owing to the small sample size.

CALR mutation was also detected in other hematological malignancies such as refractory anemia (RA; 9%), RA with ringed sideroblasts (RARS; 11%), and RA with excess blasts (RAEB; 12%). RARS is associated with marked thrombocytosis (RARS-t; 9-12%), chronic myelomonocytic leukemia (CMMoL; 3%), and atypical chronic myelogenous leukemia (aCML; 3%) [4, 5]. However, these diseases have not been studied extensively. In a

subsequent study for RARS-t, similar frequencies were not reproduced [12].

As reported in previous studies, several mutational types of *CALR* have been discovered. Among them, L367fs*46 (52 nucleotide deletion) and K385fs*47 (TTGTC insertion) accounted for >80% of the mutation types. In our study, L367fs*46 and K385fs*47 were detected in 89.3% of both the ET and PMF patients. Other mutation types, including E364fs*55, E369fs*46, and K385fs*47 (TTTGTTC insertion), were rarely detected. All five of these mutations also generated a specific C-terminus sequence that included a common peptide sequence (RMRRMR RTRRRKMRKMSPARPRTSREACLQGWTEA) reported in previous studies (Table 2). This commonly mutated sequence could be a target for diagnostic methods or therapeutic approaches. In a recent study, immunostaining was performed by using an antibody specific for the mutated amino acids of *CALR*. The researchers revealed the preferential expression of megakaryocytes for *CALR* and recommended immunostaining as a complementary method for molecular analysis [13].

The effect of *CALR* mutation on the hematological phenotype has been reported in several studies. Compared with ET patients with *JAK2* mutation, those with *CALR* mutation had lower leukocyte counts and hemoglobin values and higher platelet counts [4, 9, 10]. PMF patients with *CALR* mutations also showed similar results to those of *CALR* mutation associated with a higher platelet count and lower leukocyte count [4, 7]. In our study, a significant association with *CALR* mutations was noted only in ET patients with a lower leukocyte count. The platelet count seemed to be higher in *CALR*-mutated ET patients than in those with *JAK2* mutation, although the statistical significance was not deduced. Among PMF patients, no *CALR*-associated phenotype was detected, possibly because of the small sample size.

Although the impact of *CALR* mutation on the thrombosis risk or the clinical outcome was not analyzed in this study, it has been reported in previous studies. Rumi *et al.* [10] reported that ET patients with *CALR* mutation had a lower polycythemic transformation rate, but not a lower myelofibrotic transformation rate, compared with ET patients with *JAK2* mutation. Klampfl *et al.* [4] also demonstrated a low risk of thrombosis and longer survival for *CALR*-positive ET and PMF patients. However, a neutral effect has also been reported. For instance, Rotunno *et al.* [9] reported that *CALR* mutation had no impact on the survival or transformation to post-ET myelofibrosis. Interestingly, Tefferi *et al.* [14] attempted molecular prognostication for PMF patients based on a combination of *CALR* and additional sex combs like

1 (*ASXL1*) modifications. They found that *CALR(-)/ASXL1(+)* was a powerful risk factor representing poor prognosis. Further evaluation is required for asserting the usefulness of additional *ASXL1* genotyping for PMF.

In conclusion, *CALR* mutation is a useful diagnostic marker for *JAK2/MPL*-negative ET or PMF patients because of its mutual exclusiveness for *JAK2* mutation and the relative high frequency. The phenotypic manifestations of *CALR* mutation are different from those of *JAK2* mutation, and this fact may facilitate defining subtypes with different prognoses.

Acknowledgments

We thank KS Park, KS Lee, and TY Park for their helpful assistance with sample collection, DNA extraction, and sequencing analysis.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

REFERENCES

1. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of *JAK2* in myeloproliferative disorders. *N Engl J Med* 2005;352:1779-90.
2. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, et al. *MPLW515L* is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med* 2006;3:e270.
3. 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.
4. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013;369:2379-90.
5. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al. Somatic *CALR* mutations in myeloproliferative neoplasms with nonmutated *JAK2*. *N Engl J Med* 2013;369:2391-405.
6. Coppolino MG, Woodside MJ, Demareux N, Grinstein S, St-Arnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signaling and cell adhesion. *Nature* 1997;386:843-7.
7. Tefferi A, Lasho TL, Finke CM, Knudson RA, Ketterling R, Hanson CH, et al. *CALR* vs *JAK2* vs *MPL*-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. *Leukemia* 2014;28:1472-7.
8. Lundberg P, Karow A, Nienhold R, Looser R, Hao-Shen H, Nissen I, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood* 2014;123:2220-8.
9. Rotunno G, Mannarelli C, Guglielmelli P, Pacilli A, Pancrazzi A, Pieri L, et al. Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. *Blood* 2014;123:

- 1552-5.
10. Rumi E, Pietra D, Ferretti V, Klampfl T, Harutyunyan AS, Milosevic JD, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood* 2014;123:1544-51.
 11. Li B, Xu J, Wang J, Gale RP, Xu Z, Cui Y, et al. Calreticulin mutations in Chinese with primary myelofibrosis. *Haematologica* 2014. pii: haematol.2014.109249. [Epub ahead of print]
 12. Patnaik MM, Belachew A, Finke C, Lasho TL, Hanson CA, Tefferi A. CALR mutations are infrequent in WHO-defined refractory anemia with ring sideroblasts. *Leukemia* 2014;28:1370-1.
 13. Vannucchi AM, Rotunno G, Bartalucci N, Raugei G, Carrai V, Balliu M, et al. Calreticulin mutation-specific immunostaining in myeloproliferative neoplasms: pathogenetic insight and diagnostic value. *Leukemia* 2014; 28:1811-8.
 14. Tefferi A, Guglielmelli P, Lasho TL, Rotunno G, Finke C, Mannarelli C, et al. CALR and ASXL1 mutations-based molecular prognostication in primary myelofibrosis: an international study of 570 patients. *Leukemia* 2014;28:1494-500.