Am J Transl Res 2023;15(7):4718-4726
www.ajtr.org /ISSN:1943-8141/AJTR0150573

Original Article

Expression properties, structural features and functional analysis of CALR E381A in MPN patients

Kaihong Xu, Qunfang Ge, Yanli Zhang, Guifang Ouyang, Xiao Yan

Department of Hematology, Ningbo First Hospital, Ningbo, Zhejiang, China

Received April 5, 2023; Accepted June 24, 2023; Epub July 15, 2023; Published July 30, 2023

Abstract: Objective: To investigate the expression properties, structural features and function of CALR E381A in myeloproliferative neoplasms (MPN) patients. Methods: In this retrospective study, 435 MPN patients admitted to the Department of Hematology, Ningbo First Hospital from July 2015 to July 2021 were selected as the study subjects. Mutations in *CALR* exon 9 from genomic DNA samples were identified by PCR, followed by Sanger sequencing. The physicochemical properties of the wild-type calreticulin and the p.E381A variant, and the structural information of the p.E381A variant were analyzed by using the bioinformatics databases. Growth assay of UT-7/mpl cells with *CALR* E381A was used for the functional analysis of CALR E381A. Results: The predominant types of *CALR* variants were identified as follows: p.L367fs*46 (38.1%), p.K385fs*47 (25.8%) and p.E381A (19.6%). Notably, the frequency of the p.E381A variant (c.1142A >C) in polycythemia vera or essential thrombocythemia was significantly higher than the frequency of that as a single nucleotide polymorphism (SNP) in the East Asian population. Furthermore, *CALR* E381A coexisted with other genetic variants, of which *JAK2* V617F was more common. Bioinformatics analysis confirmed that *CALR* E381A did not change the physicochemical properties of the calreticulin protein, but did change the electrical charge, energy state and steric hindrance of amino acid residues at site 381. UT-7/mpl cells harboring *CALR* E381A overexpression did not exhibit altered cell growth, which is distinctly different from the stereotypical frameshift mutation. Conclusion: CALR E381A is not a driver mutation for the development of MPN but may be a risk SNP implying an inherited predisposition for MPN disease in East Asian populations.

Keywords: Myeloproliferative neoplasm, single nucleotide polymorphism, *CALR* E381A, *JAK2* V617F, Chinese patients

Introduction

Myeloproliferative neoplasms (MPNs), characterized by overproduction of one or several blood elements, mainly include polycythemia vera (PV), primary myelofibrosis (PMF), and essential thrombocythemia (ET). Transformation can occur between different disease subtypes, mainly including ET or PV conversion into myelofibrosis (MF), and only a very small number of ET to PV. The 10-year conversion rates of PV, ET, and PMF to acute myeloid leukemia (AML) ranged from 2% to 4%, 1%, and 10% to 20%, respectively. The progress of MPNs is slow, and disease-related complications as well as transformation to AML are the important factors affecting the quality of life and survival time of patients.

The JAK2, MPL, and CALR gene mutations are the driver gene mutations of MPNs. About 95% of patients with PV carry JAK2 mutation (JAK2V617F). The gene mutation distribution of ET patients is similar to that of PMF patients. The proportions of patients carrying JAK2, CALR and MPL mutations were 50% to 60%, 30% and 5% to 8%, respectively. In addition to the above three types of driver gene mutation, some non-driver gene mutations, such as DNMT3A, TET2, ASXL2, TP53, also play a role in the clonal evolution of MPNs. However, the mechanism by which some individuals with genetic mutations develop clonal hematopoiesis and progress to MPNs on this basis is still unclear. The somatic mutations in the Janus kinase 2 gene (*JAK2*), the gene encoding calreticulin (*CALR*), and the thrombopoietin receptor gene (*MPL*) are considered to be the most common driver mutations in MPN and serve as the molecular basis for the diagnosis of MPN.

CALR, encodes a soluble Ca^{2+}-binding protein, which influences a variety of processes in cal-

cium homeostasis, immune response, phagocytosis and signaling [1]. In 2013, mutations in exon 9 of *CALR* were identified in 60%-90% of patients with ET and PMF that were *JAK2/MPL*-mutation negative [2, 3]. These results have been repeatedly confirmed in subsequent studies [4]. Previous studies showed that the type of gene mutation could affect the disease phenotype and disease progression of MPNs. For example, patients with CALR type-1 mutation (52 bp deletion) tended to develop PMF [5], while patients with CALR type-2 mutation (5 bp insertion) were more prone to ET [6]. Moreover, it was shown that MPL mutation increased the risk of ET converting to MF in contrast to JAK2V617F and CALR mutations [7]. In addition, many studies confirmed that the increased gene mutation burden of *JAK2* V617F allele would increase the risk of PV or ET converting to MF [8]. It was considered that increased gene mutation burden would also promote the evolution of MPNs disease [9]. To date, more than 60 frameshift mutations in *CALR* have been described with all involving exon 9. The two most frequent mutations are type-1 (c.1092_1143del; p.L367fs*46) and type-2 (c.1154_1155insTTGTC; p.K385fs*47) variants [10]. It has been generally believed that mutations in *CALR* exon 9 are mutually exclusive to *JAK2* and *MPL* variants as the frequency of co-occurrence is usually below 1% [11]. This exclusivity is especially noted in PV, with only one case of coexisted *JAK2* V617F and *CALR* exon 9 mutations being reported to date [12]. This low frequency is mainly due to the fact that *CALR* mutations are rarely detected in PV. Accordingly, it has been suggested that screening for *CALR* mutations in PV is not useful [13]. Whereas, these results have been mostly found in Caucasian populations, it is unknown if there are similar results among Asian populations. Given that many investigators have shown ethnical diversity in clinical and laboratory features of MPN [14-16], it is important to determine the actual mutation profile of *CALR* exon 9 in Chinese patients in order to clarify its pathogenic potential in MPN.

In this context, we analyzed the *CALR* exon 9 of 435 Chinese patients with MPNs and defined the clinical correlates, physicochemical properties, and spatial structure of the particular type of *CALR* variant. The results of this study would provide some clinical reference for guiding treatment of patients with MPNs.

Materials and methods

Study population

In this retrospective study, MPN patients that presented at Ningbo First Hospital (Ningbo, Zhejiang, China) from July 2015 to July 2021 were selected as the study subjects. Diagnoses were established according to the 2008 World Health Organization (WHO) criteria. A total of 435 patients with MPN (256 males, 179 females; mean age 61.2 years old) were included in the study. The genomic DNA samples were extracted from bone marrow/peripheral blood and buccal mucosa. The clinical data from medical records were retrospectively reviewed. The study was approved by the Ethics Committee of the Ningbo City First Hospital (2020-R131), and informed written consent was obtained from all patients in the study.

Mutation detection

Mutations in *CALR* exon 9 were identified in the collected samples using polymerase chain reaction (PCR), followed by Sanger sequencing as reported previously [17]. The *JAK2* V617F and *MPL* W515L/K mutations were analyzed using TaqMan quantitative PCR with mutant-specific primers [18]. From 2019, the mutations were assessed by the panel-based next-generation sequencing performed on the Illumina NextSeq 500 platform (Illumina, Inc.).

Bioinformatic analysis

The protein structure of calreticulin was retrieved from the Protein Data Bank (PDB) database (structure entry 6ENY). The online bioinformatics ProtParam tool (http://www.expasy.ch/tools/protparam.html/) was used to analyze the physicochemical properties of the wild-type calreticulin and the p.E381A variant. The secondary structure of calreticulin was analyzed using the web server PDBSum database. Finally, the structural information for the p.E381A variant was visualized and analyzed using Swiss-Pdb Viewer 4.1.0.

Transfection and cell growth assay

UT-7 cell line was obtained from the Cell Resource Center, Chinese Academy of Medical Sciences (Beijing, China). The cDNA of human c-MPL (accession number NM_005373.3) from whole gene synthesis was introduced to UT-7

Table 1. Mutation profiles of *JAK2* V617F, *CALR and MPL* W515L/K in 435 patients with MPN

Diagnosis	Cases (n)	*JAK2* V617F [n (%)]	*CALR* [n (%)]	*MPL* W515L/K [n (%)]
PV	148	126 (85.1)	8 (5.4)	0
ET	191	104 (54.5)	57 (29.8)	2 (1.0)
PMF	96	47 (49.0)	32 (33.3)	3 (3.1)
Total	435	277 (63.7)	97 (22.3)	5 (1.1)

Abbreviations: MPN, myeloproliferative neoplasm; PV, polycythaemia vera; ET, essential thrombocytosis; PMF, primary myelofibrosis; n, number.

cells, using NotI/NsiI restriction site of the LV5-EF1a-GFP/Puro lentivirus vector (Gene Pharma Corp., Shanghai). The stably transfected UT-7/mpl cells were validated by Western blot with anti-MPL antibody (ab172061, Abcam). These cells were suspended in IMDM containing 10% fetal bovine serum (Gibco-Life Technologies) and 10 ng/ml recombinant human thrombopoietin (rhTPO, Peprotech). *CALR* del52 and *CALR* E381A were synthesized and separately cloned into EcoRI/HindIII-digested pcDNA3.1 (+) vector (Gene Pharma Corp., Shanghai). The recombinant plasmids and the empty vector plasmid were transferred into UT-7/mpl cells, respectively, using the cell electroporation system operator H1 (Etta Biotech Co., Ltd., Suzhou). Overexpression of construct was further verified by RT-PCR. UT-7/mpl cells harboring overexpression of constructs in the absence of TPO for 5 days were assessed with a TC20 Automated Cell Counter (Bio-Rad) through Trypan Blue exclusion. UT-7/mpl cells harboring the empty vector plasmid was served as a control.

Statistical analysis

Using SPSS software version 22.0, the *Z* test was used to compare the frequency of mutated gene between patients and the healthy population. The probability of a gene variant detected in <5 patients was directly calculated according to the principle of binomial distribution. α=0.05 was taken as the statistical test level.

Results

Identification of CALR mutations in patients with MPN

A total of 435 patients with *BCR/ABL*-negative MPN at initial diagnosis were included in this study, including 148 PV, 191 ET, and 96 PMF. Mutation profiles of *JAK2* V617F, *CALR and MPL* W515L/K in the 435 patients with MPN are shown in **Table 1**. In 148 patients with PV, there were 126 patients with *JAK2* V617F mutations and 8 patients with *CALR* Exon 9 mutations, and there was no patient with *MPL* W515L/K mutations. In 191 patients with ET, there were 104 patients with *JAK2* V617F mutations, 57 patients with *CALR* Exon 9 mutations, and 2 patients with *MPL* W515L/K mutations. In 96 patients with PMF, there were 47 patients with *JAK2* V617F mutations, 32 patients with *CALR* Exon 9 mutations, and 3 patients with *MPL* W515L/K mutations. Of the 97 patients with *CALR* Exon 9 mutations, the proportion of p.L367fs*46 (type-1), p.K385fs*47 (type-2) and p.E381A (c.1142A >C) were 38.1%, 25.8% and 19.6%, respectively, which were the predominant mutation types, as shown in **Figure 1**.

Patients with the CALR E381A

CALR E381A was identified in 19 patients with MPN, including 8 cases of PV, 7 cases of ET, 3 cases of PMF and 1 case of CML (*BCR/ABL* was detected 4 years after the initial ET diagnosis). Clinical and laboratory features of patients with *CALR* E381A are shown in **Table 2**. Notably, *CALR* E381A could coexist with various gene alterations, of which *JAK2* V617F was more common. The co-occurrence of *CALR* E381A and *JAK2* V617F mutation was identified in 9 cases. The frequencies of *CALR* E381A in *JAK2* V617F-positive PV, PV and ET were 3.2% (4/126), 5.4% (8/148), and 3.7% (7/191), respectively. *CALR* E381A is regarded as a single nucleotide polymorphism (SNP: rs14388-0510) with a frequency of 1.45% in the East Asian population according to the National Center for Biotechnology Information (NCBI) Allele Frequency Aggregator (ALFA) database (https://www.ncbi.nlm.nih.gov/snp/rs14388-0510#frequency_tab). Compared with the frequency, there was a significantly higher frequency of *CALR* E381A in patients with PV (Z=4.02, P<0.001), or patients with ET (Z=2.60, P<0.05). Sequencing analysis of buccal swab-isolated DNA revealed that *CALR* E381A was a germline variant.

Structural features of CALR E381A based on bioinformatic analysis

The c.1142A >C variant is a missense mutation occurring in the C-terminus of calreticulin and

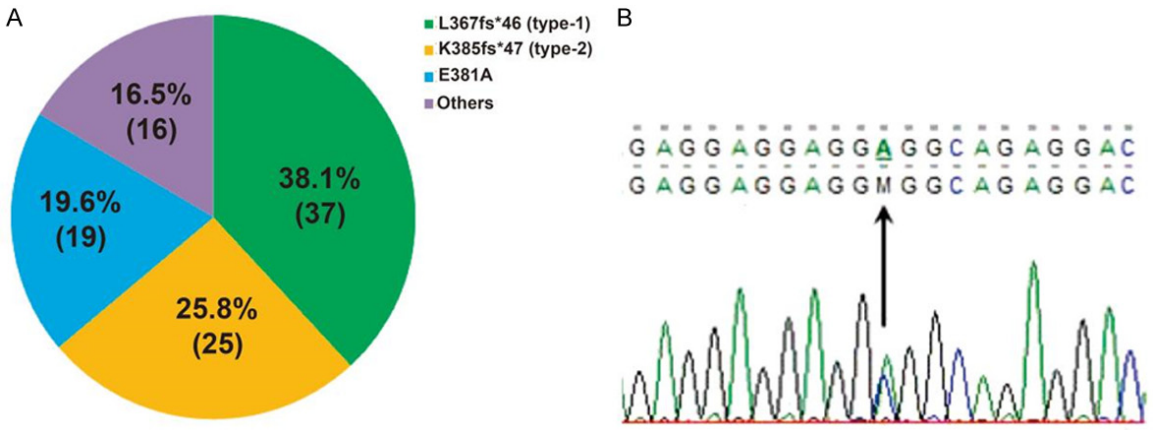

Figure 1. *CALR* E381A in myeloproliferative neoplasm. A. Type of *CALR* exon 9 mutations identified in 97 patients with myeloproliferative neoplasm. B. Sequence analysis of the c.1142A >C (p.E381A) mutation in *CALR* exon 9.

Table 2. Clinical and laboratory features of patients with *CALR* E381A

Case	Gender/Age	Diagnosis	Coexistence	WBC (10^9/L)	HB (g/L)	PLT (10^9/L)	Thrombosis related events	Splenomegaly
1	M/53	PV	*JAK2* V617F	10.3	236.	751	N	Y
2	F/55	PV	*JAK2* V617F	9.4	191	306	Cerebral thrombosis	N
3	M/59	PV	*JAK2* V617F	14.9	212	273	N	Y
4	F/69	PV	*JAK2* V617F	8.5	180	403	Myocardial infarction	N
5	M/53	PV	N	4.02	191	119	Calf vein thrombosis	N
6	M/57	PV	N	6.41	196	173	N	N
7	M/65	PV	N	5.92	187	178	N	Y
8	M/50	PV	N	4.04	198	212	N	N
9	M/71	ET	*JAK2* V617F	9.73	152	861	N	N
10	F/30	ET	*JAK2* V617F	9.5	132	740	Habitual abortion	Y
11	F/47	ET	*JAK2* V617F	9.3	125	613	N	N
12	F/32	ET	*JAK2* V617F	10.5	153	1544	N	Y
13	F/22	ET	p.K385fs*47 (type-2)	10.3	191	751	N	Y
14	M/73	ET	*MPL* W515L	4.42	129	1082	Intestinal bleeding	Y
15	M/41	ET	N	11.2	141	1474	N	Y
16	F/69	PMF	N	15.1	76	406	Pulmonary embolism	Y
17	F/54	PMF	*JAK2* V617F	16.5	116	711	N	Y
18	M/62	PMF	*NRAS* (p.Gly12Ser)	29.9	107	116	N	Y
19	M/50	CML (ET)	*BCR/ABL*	13.82	103	424	N	Y

leads to the Glu381Ala amino acid substitution (**Figure 2A**). ProtParam tool showed that this point mutation did not significantly change the physicochemical properties of the protein compared with that of the wild-type one, as seen in **Table 3**. Both proteins were acidic, negatively charged, hydrophilic and unstable.

Swiss-Pdb Viewer showed that the formation of intermolecular hydrogen bonds did not change, but the space occupied by the amino acid side chain became smaller with the mutation, resulting in reduced steric hindrance (**Figure 2B**). Concomitantly, both the Van der Waals force and the energy decreased at amino acid 381 of calreticulin (**Figure 2C**; **Table 4**). Glu is a negatively charged, polar, hydrophilic amino acid. The substitution of Ala for the Glu residue resulted in a neutral, nonpolar, hydrophobic position with a lower energy state. This was predicted to be possibly deleterious based on its combined annotation-dependent depletion (CADD) score of 20.4.

Functional analysis of CALR E381A

We generated the UT-7/mpl cell line by lentivirus transduction, which stably expressed TPO

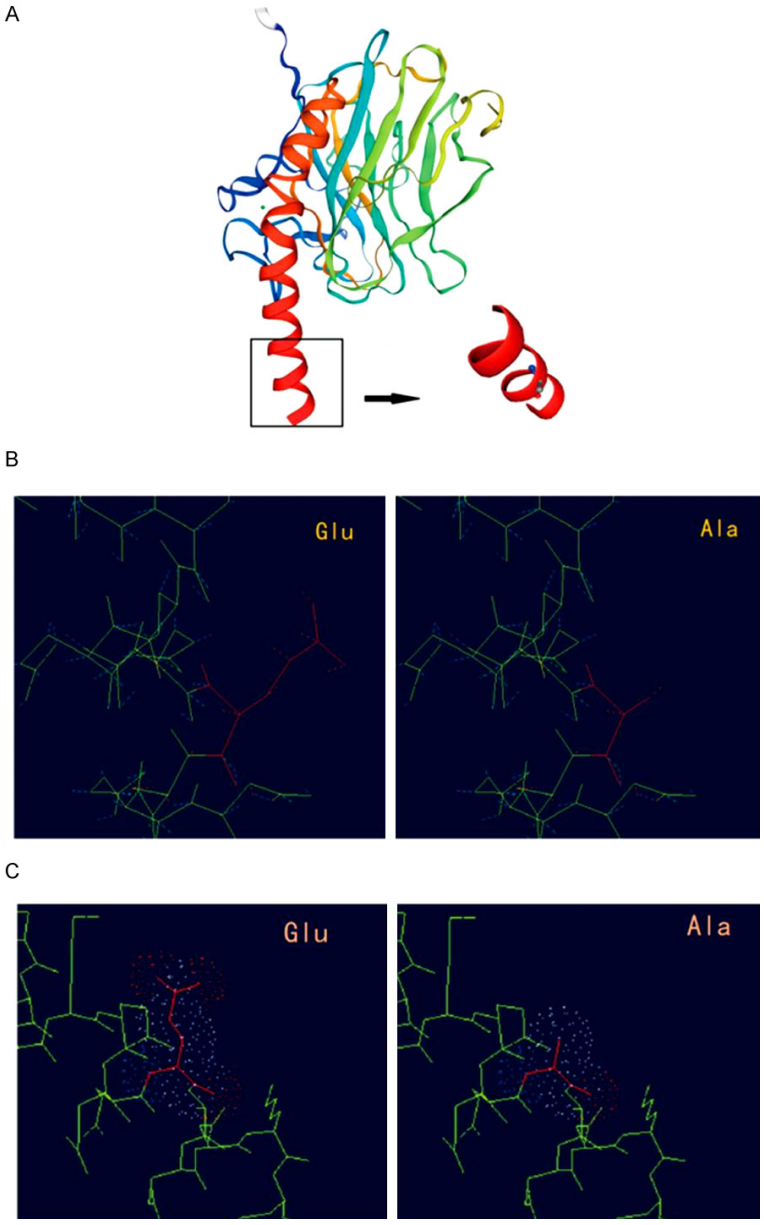

Figure 2. Structural features of *CALR* E381A. A. The E381A mutation occurred in the C-terminal domain of calreticulin. B. Presentation of residues with forces vectors before and after the E381A substitution. C. Glu and Ala at amino acid 381 of calreticulin depicted as a Van der Waals surface (carbon atoms, grey; oxygen atoms, red; nitrogen atoms, blue).

receptor (MPL) and could maintain proliferation in a TPO-dependent manner. When UT-7/mpl cells with overexpression of constructs were cultured for 5 days in TPO-free medium, the number of viable cells harboring *CALR* E381A or control decreased gradually, only cells harboring *CALR* del52 showed the proliferation of live cells (**Figure 3**). These results indicate that *CALR* E381A was not sufficient to alter growth characteristics of UT-7/mpl as *CALR* del52 did.

Discussion

Due to the mutation of genes in the human body, the active proliferation of one or more lineages of myeloid cells mainly occurs in patients with myeloproliferative tumors. The findings of clonal molecular markers, such as JAK2, MPL and CALR, are of great significance for understanding the molecular pathogenesis, diagnosis and treatment of MPNs. Detection of genetic mutations has become the main diagnostic method for MPNs. The prevalence of JAK2V617F mutation is as high as 95% in PV patients and about 50%-60% in ET and PMF patients. The mutation of JAK2V617F gene occurs at 1849th nucleotide in No. 14 exon of the JAK2 gene, changing from guanine G to thymine T, resulting in the 617th valine codon of the pseudokinase domain (JH2) being replaced by phenylalanine acid, which eliminates the negative regulation of tyrosine kinase activity. At present, JAK2V617F gene mutation has become a diagnostic marker of MPN. Moreover, the MPL gene, located at 1p34, encodes a TPO receptor through JAK-STAT signaling and plays an important role in the proliferation and differentiation of megakaryocyte, and platelet maturation. It was found in patients with ET and PMF that the mutation of this gene occurred at the 515th amino acid changing from tryptophan (W) to leucine (L) or lysine (K), which was presented as MPL-W515L/K. Rare mutations include MPLW515A/R and MPLS505N. These mutations appear as gain-of-function mutations. At present, many studies at home and abroad pointed out that patients with different driver

Table 3. Physicochemical properties of the wild-type calreticulin and the p.E381A variant

Parameters		Wild type	p.E381A
Number of amino acids		417	
Molecular weight		48141.56	48083.82
Theoretical pI		4.29	4.3
Number of negatively charged residues		109	108
Number of positively charged residues		50	50
Total number of atoms		6598	6592
Estimated half-life	(Mammalian reticulocytes, *in vitro*)	30 hours	
	(Yeast, *in vivo*)	>20 hours	
	(*Escherichia coli, in vivo*)	>10 hours	
Instability index		47.56	46.78
Aliphatic index		59.14	59.38
Grand average of hydropathicity		-1.104	-1.092

Table 4. Energy computations for Glu and Ala at amino acid 381 of calreticulin determined using the GROMOS96 simulation software of the Swiss-Pdb Viewer

Residue	Bond (KJ/mol)	Angle (KJ/mol)	Torsion (KJ/mol)	Improper torsion (KJ/mol)	Total (KJ/mol)
Glu	0.969	2.991	3.332	1.62	8.912
Ala	0.518	1.196	0.942	1.617	4.273

Note: In this simulation, all computations were done in vacuo without reaction field.

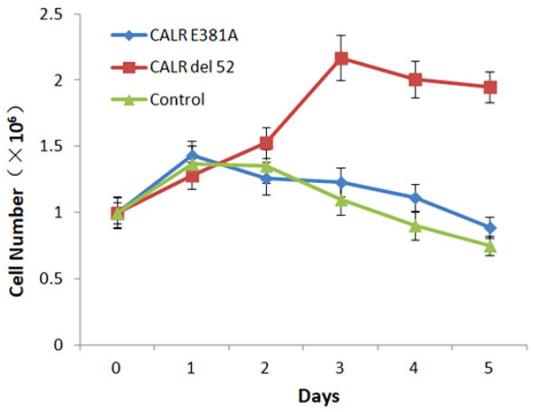

Figure 3. Growth assay of UT-7/mpl cells with *CALR* E381A. Live cells with *CALR* E381A were counted everyday using an automated cell counter in the absence of TPO compared with UT-7/mpl cells harboring *CALR* del52 or empty plasmid.

gene mutations had significantly different disease characteristics [19]. For example, patients with positive CALR mutation had a younger age of onset, a lower risk of thrombosis, and a relatively better prognosis in contrast to patients with JAK2V617F mutations. It is characterized by a relatively better prognosis [20]. Previous studies mostly believed that MPNs patients only showed mutations in one of the above three driver genes, and the driver genes were mutually exclusive [21]. However, in recent years, MPNs cases with co-expression of JAK2V617F/CALR and JAK2V617F/MPL have been reported [22]. Double mutation of driver genes suggests that the driver gene may not alone cause the pathogenesis of MPN, and the mutations at different sites may promote each other and cause the occurrence of disease together.

CALR gene, located at 19p13, is a highly conserved endoplasmic reticulum molecular chaperone, mainly involved in the regulation of calcium ion balance, protein folding and processing. It was found CALR frameshift mutations existed in JAK2/MPL-negative ET and PMF patients (50%-60% ET and 75% PMF) [23]. This mutation is an insertion/deletion of exon 9 and results in a specific C-carboxy terminus lacking the endoplasmic reticulum retention sequence KDEL, leading to altered biological function. It was also found that the specific C-carboxy terminus blocked the P-domain, which prompted the N-domain in mutant CALR to preferentially interact with MPL, further inducing JAK2 activation and cytokine-independent growth [24]. Constitutive activation of the receptor by this mutant molecular chaperone is

considered as a novel molecular mechanism of cellular transformation in MPNs [25]. There are more than 50 CALR frameshift mutations reported in previous literature. The most common mutation types are the type-1 variant (p.L367fs*46) caused by a 52-base deletion (C.1099_1150del52bp) and type-2 variant (p.K385fs*47) caused by a 5-base TTGTC insertion (c.1154_1155insTTGTC).

We unexpectedly found that *CALR* E381A was not rare in MPN. As a SNP, *CALR* E381A has been previously identified in East Asian patients with ET [26]. In this study, the frequency of *CALR* E381A in patients with PV or ET was significantly higher than that as a SNP, especially in the PV patients, with a frequency as high as 5.4%.

It is noteworthy that C*ALR* E381A not only coexists with *JAK2* V617F in PV, but also appears to be a "collaborator" that could coexist with *BCR/ABL, MPL* W515L*, NRAS* and even a frameshift mutation of its own gene, according to the genetic assay results for 19 patients. Based on previous research [27], it seems reasonable to believe that *CALR* E381A as a germline mutation, is an early event, while other mutations including *JAK2* V617F, *BCR/ABL* and frameshift mutations are later events in MPN pathogenesis.

Generally, it is considered that frameshift mutations in *CALR* are pathogenic for leading to a change in the C-terminus peptide with the loss of most of the acidic domain and the KDEL motif [28]. *CALR* del52, as a representative of stereotypical frameshift mutations in *CALR,* is known to abnormally bind with and activate *MPL* which continues to act on JAK2/STAT5 signaling as well as on the MAPK pathway, justifying the cell hyper-proliferation [29]. Structurally, *CALR* E381A is a point mutation that maintains C-terminus and KDEL motif in *CALR*, which is distinctly different from the stereotypical frameshift pattern. Cell proliferation assay also confirmed that *CALR* E381A showed a failure of cell growth without TPO supplementation, whereas the situation from frameshift *CALR* 52del was rather different. We suppose that *CALR* E381A may not be sufficient to be a driver mutation, but contribute to clonal evolution events, like a predisposing factor that offers "fertile ground" for pathogenic somatic mutations in the early pathogenesis of MPN [30]. Given that the effects of *CALR* E381A was investigated by transient overexpression *in vitro*, further studies are needed to determine the long-term role of *CALR* E381A *in vivo*.

Bioinformatics analysis showed that *CALR* E381A changed the electrical charge, steric hindrance and energy state at position 381 in the C-domain of calreticulin, which is an important amino acid site and has been reported of anomalies repeatedly, even though its function has not yet been determined [31-33]. The property of the acidic C-domain of calreticulin binding multiple calcium ions with low affinity has been corroborated [34]. Thus, it makes sense to assume that *CALR* E381A may alter the affinity of the C-domain binding with Ca^{2+}, thereby affecting a range of biological processes. The detailed mechanism underlying this process is worth of exploration.

In conclusion, *CALR* E381A not only has a significantly higher frequency in patients with PV or ET than that as a SNP, but also is able to coexist with various genetic variants including *JAK2* V617F. It may serve as a marker that would be beneficial in the diagnosis of MPN and a risk SNP implying an inherited predisposition for the development of MPN in East Asian populations. However, our study had some limitations. First, a small number of patients were included in the study. Second, the effects of other concomitant somatic mutations were not considered. Third, the animal experiments of MPN were not conducted. Finally, a comparison with *JAK2*-mutated MPN was not performed. More experiments are needed in future for further confirmation.

Disclosure of conflict of interest

None.

Address correspondence to: Xiao Yan, Department of Hematology, Ningbo First Hospital, No. 59, Liuting Street, Haishu District, Ningbo 315000, Zhejiang, China. Tel: +86-0574-87085588; Fax: +86-0574-87291583; E-mail: yanxiao_@outlook.com

References

[1] Fucikova J, Spisek R, Kroemer G and Galluzzi L. Calreticulin and cancer. Cell Res 2021; 31: 5-16.

[2] Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, Them NC, Berg T,

Gisslinger B, Pietra D, Chen D, Vladimer GI, Bagienski K, Milanesi C, Casetti IC, Sant'Antonio E, Ferretti V, Elena C, Schischlik F, Cleary C, Six M, Schalling M, Schonegger A, Bock C, Malcovati L, Pascutto C, Superti-Furga G, Cazzola M and Kralovics R. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 2013; 369: 2379-2390.

[3] Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, Avezov E, Li J, Kollmann K, Kent DG, Aziz A, Godfrey AL, Hinton J, Martincorena I, Van Loo P, Jones AV, Guglielmelli P, Tarpey P, Harding HP, Fitzpatrick JD, Goudie CT, Ortmann CA, Loughran SJ, Raine K, Jones DR, Butler AP, Teague JW, O'Meara S, McLaren S, Bianchi M, Silber Y, Dimitropoulou D, Bloxham D, Mudie L, Maddison M, Robinson B, Keohane C, Maclean C, Hill K, Orchard K, Tauro S, Du MQ, Greaves M, Bowen D, Huntly BJP, Harrison CN, Cross NCP, Ron D, Vannucchi AM, Papaemmanuil E, Campbell PJ and Green AR. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 2013; 369: 2391-2405.

[4] Wiriyaukaradecha K, Nimsanor S, Tantirukdham N, Tongsom J, Bunyoo C, Soonklang K, Sritana N and Auewarakul C. Study of CALR, MPL, and c-kit gene mutations in Thai patients with JAK2 V617F negative myeloproliferative neoplasms. Asian Pac J Cancer Prev 2022; 23: 1671-1678.

[5] Rosso V, Petiti J, Bracco E, Pedrola R, Carnuccio F, Signorino E, Carturan S, Calabrese C, Bot-Sartor G, Ronconi M, Serra A, Saglio G, Frassoni F and Cilloni D. A novel assay to detect calreticulin mutations in myeloproliferative neoplasms. Oncotarget 2017; 8: 6399-6405.

[6] Nie YB, Sun M, He CK, Ju MK, Zhou FL, Wu SY, Zhou Y, Liu L, Shen H, Huang TT, Liu P, Xu Y, Shao L and Zuo XL. ASXL1 mutations in Chinese patients with essential thrombocythemia. Exp Ther Med 2018; 15: 4149-4156.

[7] Wang Y, Ran F, Lin J, Zhang J and Ma D. Genetic and clinical characteristics of patients with philadelphia-negative myeloproliferative neoplasm carrying concurrent mutations in JAK2V617F, CALR, and MPL. Technol Cancer Res Treat 2023; 22: 15330338231154092.

[8] Kim SY, Im K, Park SN, Kwon J, Kim JA and Lee DS. CALR, JAK2, and MPL mutation profiles in patients with four different subtypes of myeloproliferative neoplasms: primary myelofibrosis, essential thrombocythemia, polycythemia vera, and myeloproliferative neoplasm, unclassifiable. Am J Clin Pathol 2015; 143: 635-644.

[9] Shahin OA, Chifotides HT, Bose P, Masarova L and Verstovsek S. Accelerated phase of myeloproliferative neoplasms. Acta Haematol 2021; 144: 484-499.

[10] Di Buduo CA, Abbonante V, Marty C, Moccia F, Rumi E, Pietra D, Soprano PM, Lim D, Cattaneo D, Iurlo A, Gianelli U, Barosi G, Rosti V, Plo I, Cazzola M and Balduini A. Defective interaction of mutant calreticulin and SOCE in megakaryocytes from patients with myeloproliferative neoplasms. Blood 2020; 135: 133-144.

[11] He R, Hanson CA, Chen D, Oliveira JL, Pardanani A, Reichard KK and Viswanatha DS. Not all CALR mutations are created equal. Leuk Lymphoma 2015; 56: 2482-2483.

[12] Xu N, Ding L, Yin C, Zhou X, Li L, Li Y, Lu Q and Liu XL. A report on the co-occurrence of JAK2V617F and CALR mutations in myeloproliferative neoplasm patients. Ann Hematol 2015; 94: 865-867.

[13] Chauveau A, Nibourel O, Tondeur S, Paz DL, Mansier O, Paul F, Wemeau M, Preudhomme C, Lippert E and Ugo V; French Intergroup of Myeloproliferative Neoplasms. Absence of CALR mutations in JAK2-negative polycythemia. Haematologica 2017; 102: e15-e16.

[14] Li B, Xu J, Wang J, Gale RP, Xu Z, Cui Y, Yang L, Xing R, Ai X, Qin T, Zhang Y, Zhang P and Xiao Z. Calreticulin mutations in Chinese with primary myelofibrosis. Haematologica 2014; 99: 1697-1700.

[15] Wu Z, Zhang X, Xu X, Chen Y, Hu T, Kang Z, Li S, Wang H, Liu W, Ma X and Guan M. The mutation profile of JAK2 and CALR in Chinese Han patients with Philadelphia chromosome-negative myeloproliferative neoplasms. J Hematol Oncol 2014; 7: 48.

[16] Xu Z, Gale RP, Zhang Y, Qin T, Chen H, Zhang P, Zhang T, Liu L, Qu S and Xiao Z. Unique features of primary myelofibrosis in Chinese. Blood 2012; 119: 2469-2473.

[17] Tefferi A, Lasho TL, Finke CM, Knudson RA, Ketterling R, Hanson CH, Maffioli M, Caramazza D, Passamonti F and Pardanani A. CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. Leukemia 2014; 28: 1472-1477.

[18] Wu Z, Zhang Y, Zhang X, Xu X, Kang Z, Li S, Zhang C, Su B and Guan M. A multiplex snapback primer system for the enrichment and detection of JAK2 V617F and MPL W515L/K mutations in philadelphia-negative myeloproliferative neoplasms. Biomed Res Int 2014; 2014: 458457.

[19] Tefferi A. Myeloproliferative neoplasms: a decade of discoveries and treatment advances. Am J Hematol 2016; 91: 50-58.

[20] Michiels JJ, Berneman Z, Schroyens W and De Raeve H. Changing concepts of diagnostic criteria of myeloproliferative disorders and the molecular etiology and classification of myeloproliferative neoplasms: from Dameshek 1950 to Vainchenker 2005 and beyond. Acta Haematol 2015; 133: 36-51.

[21] Vannucchi AM and Guglielmelli P. Molecular prognostication in Ph-negative MPNs in 2022. Hematology Am Soc Hematol Educ Program 2022; 2022: 225-234.
[22] Vainchenker W and Kralovics R. Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. Blood 2017; 129: 667-679.
[23] Frawley T, O'Brien CP, Conneally E, Vandenberghe E, Percy M, Langabeer SE and Haslam K. Development of a targeted next-generation sequencing assay to detect diagnostically relevant mutations of JAK2, CALR, and MPL in myeloproliferative neoplasms. Genet Test Mol Biomarkers 2018; 22: 98-103.
[24] Araki M and Komatsu N. Novel molecular mechanism of cellular transformation by a mutant molecular chaperone in myeloproliferative neoplasms. Cancer Sci 2017; 108: 1907-1912.
[25] Araki M, Yang Y, Masubuchi N, Hironaka Y, Takei H, Morishita S, Mizukami Y, Kan S, Shirane S, Edahiro Y, Sunami Y, Ohsaka A and Komatsu N. Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. Blood 2016; 127: 1307-1316.
[26] Lim KH, Chang YC, Gon-Shen Chen C, Lin HC, Wang WT, Chiang YH, Cheng HI, Su NW, Lin J, Chang YF, Chang MC, Hsieh RK, Kuo YY and Chou WC. Frequent CALR exon 9 alterations in JAK2 V617F-mutated essential thrombocythemia detected by high-resolution melting analysis. Blood Cancer J 2015; 5: e295.
[27] Grinfeld J, Nangalia J, Baxter EJ, Wedge DC, Angelopoulos N, Cantrill R, Godfrey AL, Papaemmanuil E, Gundem G, MacLean C, Cook J, O'Neil L, O'Meara S, Teague JW, Butler AP, Massie CE, Williams N, Nice FL, Andersen CL, Hasselbalch HC, Guglielmelli P, McMullin MF, Vannucchi AM, Harrison CN, Gerstung M, Green AR and Campbell PJ. Classification and personalized prognosis in myeloproliferative neoplasms. N Engl J Med 2018; 379: 1416-1430.
[28] Liu P, Zhao L, Kroemer G and Kepp O. Secreted calreticulin mutants subvert anticancer immunosurveillance. Oncoimmunology 2020; 9: 1708126.
[29] Chachoua I, Pecquet C, El-Khoury M, Nivarthi H, Albu RI, Marty C, Gryshkova V, Defour JP, Vertenoeil G, Ngo A, Koay A, Raslova H, Courtoy PJ, Choong ML, Plo I, Vainchenker W, Kralovics R and Constantinescu SN. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. Blood 2016; 127: 1325-1335.
[30] Lundberg P, Nienhold R, Ambrosetti A, Cervantes F, Perez-Encinas MM and Skoda RC. Somatic mutations in calreticulin can be found in pedigrees with familial predisposition to myeloproliferative neoplasms. Blood 2014; 123: 2744-2745.
[31] Belcic Mikic T, Pajic T and Sever M. CALR mutations in a cohort of JAK2 V617F negative patients with suspected myeloproliferative neoplasms. Sci Rep 2019; 9: 19838.
[32] Smaili W, Doubaj Y, Laarabi FZ, Lyahyai J, Kerbout M, Mikdame M and Sefiani A. CALR gene mutational profile in myeloproliferative neoplasms with non-mutated JAK2 in Moroccan patients: a case series and germline in-frame deletion. Curr Res Transl Med 2017; 65: 15-19.
[33] Pietra D, Rumi E, Ferretti VV, Di Buduo CA, Milanesi C, Cavalloni C, Sant'Antonio E, Abbonante V, Moccia F, Casetti IC, Bellini M, Renna MC, Roncoroni E, Fugazza E, Astori C, Boveri E, Rosti V, Barosi G, Balduini A and Cazzola M. Differential clinical effects of different mutation subtypes in CALR-mutant myeloproliferative neoplasms. Leukemia 2016; 30: 431-438.
[34] Wijeyesakere SJ, Gafni AA and Raghavan M. Calreticulin is a thermostable protein with distinct structural responses to different divalent cation environments. J Biol Chem 2011; 286: 8771-8785.