Clinical importance of different calreticulin gene mutation types in wild-type *JAK2* essential thrombocythemia and myelofibrosis patients

The Janus kinase 2 (*JAK2*) V617F mutation (*JAK2* V617F), *JAK2* exon 12 mutations and myeloproliferative leukemia virus oncogene W515L/K mutation (*MPL* W515L/K) have become three major molecular diagnosis criteria for myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) from 2005.[1] However, diagnosing MPNs with non-mutated *JAK2* and *MPL* remains a major diagnostic challenge.[2-4] Some recent studies have reported calreticulin (*CALR*) gene mutations in patients with non-mutated *JAK2* V617F MPNs.[5-7] There are some distinct mutation types in MPN subtypes, but the differences in the clinical significance and prognosis among the different mutation types are obscure.[8-10] Here, we report our data on *CALR* mutation in wild-type (wt) *JAK2* MPN on patients. It should also be mentioned that this is undoubtedly the first report regarding *CALR* mutations in Chinese MPN patients.

From January 2008 to December 2013, bone marrow or peripheral blood samples from 301 MPNs patients were collected in the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province, China, including ET (n=222), PV (n=37), PMF (n=33), post-ET MF (PET-MF; n=6), and post-PV MF (PPV-MF; n=3). We also obtained bone marrow samples from 174 patients with other myeloid neoplasms including: myelodysplastic syndrome (MDS; n=8), chronic myelogeneous leukemia (CML; n=55), acute myeloid leukemia (AML; n=104), and chronic myelomonocytic leukemia (CMML; n=7), as well as peripheral blood samples from 121 healthy controls. All participants provided their informed consent. Genomic PCR combined with direct and cloning sequencing was applied to screen *CALR* mutations.

A total of 24.3% (73 of 301) patients with MPNs were found harboring *CALR* mutations. The *CALR* mutation was detected in 31.1% (69 of 222) and 12.1% (4 of 33) of patients with ET and PMF, respectively (Figure 1A). Moreover, *CALR* mutations were found in 57.0% (69 of 121) ET patients with wt *JAK2* and 30.8% (4 of 13) PMF patients with wt *JAK2*. No *CALR* mutation in patients with PV, PET-MF, PPV-MF (Figure 1A) was found. The *CALR* mutations have multiple deletions or insertions including: L367fs*46 (33 of 74; 44.6%), K385fs*47 (25 of 74; 33.8%), K368fs*51 (3 of 74; 4.1%), Q365fs*50 (3 of 74; 4.1%), E364fs*49 (2 of 74; 2.7%), K374fs*56 (2 of 74; 2.7%), L367fs*48 (1 of 74; 1.4%), Q365fs*48 (1 of 74; 1.4%), E364fs*55 (1 of 74; 1.4%), K375fs*48 (1 of 74; 1.4%), K375fs*55 (1 of 74; 1.4%), and K377fs*50 (1 of 74; 1.4%).

These patients with MPNs were simultaneously examined for the presence of other gene mutations. PV patients were screened for *JAK2* V617F and *JAK2* exon 12 mutations, while ET and MF patients were screened for *JAK2* V617F and *MPL* W515L/K mutation. Among the total 301 patients with MPNs, 52.2% (157 of 301) were found to harbor *JAK2* V617F mutation. Among the 222 patients with ET and 37 patients with PV, 0.9% (2 of 222) were found to harbor MPL W515L/K mutations and 2.7% (1 of 37) to harbor *JAK2* exon 12 mutation, respectively (Figure 1B). *JAK2* V617F, *JAK2* exon 12 mutation, *MPL* W515L/K mutations

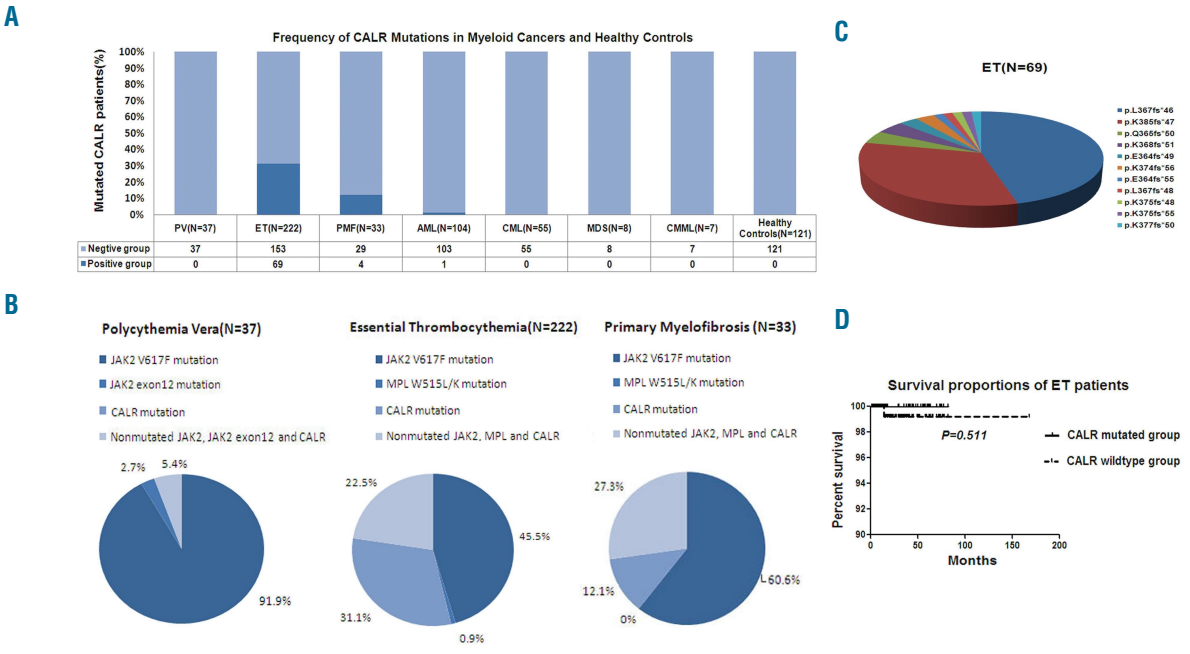

Figure 1. (A) Frequency of *CALR* mutations in myeloid neoplasms and healthy control. The *CALR* mutation was detected in 31.1% (69 of 222) and 12.1% (4 of 33) of ET and PMF patients, respectively. Approximately 1% of patients (1 of 104) with AML were found to harbor *CALR* mutation. No *CALR* mutations were found in patients with PV, other myeloid neoplasms or healthy controls. (B) The distribution of *JAK2*, *MPL*, and *CALR* mutations in the three classical entities of myeloproliferative neoplasms, including ET, PV and PMF. (C) Different mutation types in ET patients with *CALR* mutation. Most of the mutation types of *CALR* were L367fs*46(31/69,44.9%) and K385fs*47(23/69,33.3%). (D) There was no significant difference in overall survival between ET patients with *CALR* mutation and *JAK2* V617F (*P*=0.511).

and *CALR* mutations were found exclusively in these MPNs patients.

We also screened *CALR* mutations in 104 AML patients, 55 CML patients, 7 CMML patients, and 8 MDS patients (including 5 refractory cytopenia with multilineage dysplasis, 2 refractory anemia with excess blasts, and one refractory anemia) to investigate whether *CALR* mutations were present in other myeloid neoplasms. Although most of these patients had negative results, one AML patient (59-years old, male, M2 subtype) was found to harbor *CALR* mutation (L367fs*46) without *JAK2* V617F and *MPL* W515L/K mutations (Figure 1A). This patient had no previous history of MPN or MDS, Fms-related tyrosine kinase 3 internal tandem duplication, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog mutation, nucleophosmin mutation. CCAAT/enhancer binding protein alpha mutation was all negative and cytogenetics analysis showed normal karyotype. In addition, no *CALR* mutation was detected in the 121 healthy controls (Figure 1A).

For mutation types, a total of 12 distinct variants of *CALR* mutation, including 11 deletions and one insertion, were identified in our patients. L367fs*46, which resulted from a 52-bp deletion, and K385fs*47, which resulted from a 5-bp insertion, were the most frequent *CALR* mutations. The two mutations accounted for 44.6% (33 of 74) and 33.8% (25 of 74) in all cases with mutant *CALR*, respectively. For ET patients, the two mutations were 14.0% (31 of 222) and 10.4% (23 of 222), respectively. For PMF patients, the two mutations were 3.0% (1 of 33) and 6.1% (2 of 33), respectively. There was no significant difference in the two mutation types between patients with ET and PMF (P=0.137 and P=0.645, respectively). Moreover, the two mutations

Table 1. Clinical features of essential thrombocythemia and primary myelofibrosis patients with *CALR* and *JAK2* mutation.

		Essential thrombocythemia (n=222)					Primary myelofibrosis (n=33)				
		mutant *CALR*	wt *CALR*	*JAK2* V617F	*P**	*P*#	mutant *CALR*	wt *CALR*	*JAK2* V617F	*P**	*P*#
N. of patients (male: female)		69(31:38)	153(74:79)	101(49:52)	0.562	0.645	4(2:2)	29(12:17)	20(9:11)	1.0	1.0
Age (years)		50(19-80)	60(17-95)	63(21-95)	0.158	< 0.001	52(50-80)	53(28-77)	59(28-79)	0.353	0.626
WBC (×10⁹/L)		7.9(4.8-10.9)	11.7(3.1-111.3)	14(3.1-111.3)	< 0.001	< 0.001	10.12(1.5-18.74)	9.7(1.4-102.1)	16.2(3.9-102.1)	0.807	0.612
Hct (%)		41(30-47)	40.5(1.9-63)	43(1.9-59)	0.140	0.002	42(26.2-58)	42.1(17-64)	44(34-64)	0.936	0.597
Hb (g/L)		137(104-159)	132(57-205)	144(66-205)	0.334	0.002	66.5(47-86)	140(58-221)	150(100-211)	0.032	0.001
PLT (×10⁹/L)		983(317-1441)	630(43-1881)	515(43-1576)	0.156	< 0.001	174(92-256)	253(19-1215)	613(199-1215)	0.292	0.099
Thrombotic event(%)		0	3.3	1.0	0.344	1.0	0	0	0	–	–
Prognosis(n.)											
ET	PMF				< 0.001	< 0.001				0.248	0.352
Low risk	Low risk	48	70	27	–	–	0	8	4	–	–
Intermediate risk	Intermediate risk-1	20	86	48	–	–	2	14	11	–	–
–	Intermediate risk-2	–	–	–			2	7	5	–	–
High risk	High risk	1	40	26	–	–	0	0	0	–	–
OS(months)		44(5-82)	30(1-168)	33(2-168)	0.511	1.0	28(6-46)	21(4-62)	29(4-74)	0.729	1.0

*ET: essential thrombocythemia; PMF: primary myelofibrosis; WBC: white blood cell count; Hct: hematocrit; Hb: hemoglobin; PLT: platelets; RBC: red blood cell count; OS: overall survival; wt: wild-type. P*describes differences between mutant CALR and wt CALR in ET and PMF patients' group. P# describes differences between mutant CALR and JAK2 V617F in ET and PMF patients' group.*

Table 2. Clinical characteristics of essential thrombocythemia patients with different types of *CALR* and *JAK2* mutation.

	Essential thrombocythemia (N=222)									
	L367fs*46 (A)	K385fs*47 (B)	Other mutation of *CALR*(C)	*JAK2* V617F (D)	*P* (A) *vs.* (B)	*P* (A) *vs.* (C)	*P* (A) *vs.* (D)	*P* (B) *vs.* (C)	*P* (B) *vs.* (D)	*P* (C) *vs.* (D)
N. of patients (male:female)	31(12:19)	23(13:10)	15(6:9)	101(49:52)	0.194	0.933	0.338	0.319	0.488	0.538
Age(years)	55(24-77)	40(19-76)	50(34-72)	63(21-95)	0.065	0.742	0.020	0.110	<0.001	0.222
WBC (×10⁹/L)	7.9(4.3-17)	6.84(5.1-10.66)	10.5(5.2-13.1)	14(3.1-111.3)	0.159	0.732	<0.001	0.218	<0.001	0.139
Hct (%)	38.2(32-44.3)	40.1(30-47)	38.5(28-43)	43(1.9-59)	0.298	0.417	0.001	0.244	0.066	0.158
Hb (g/L)	123(101-147)	127(104-159)	123(87-156)	144(66-205)	0.671	0.829	0.002	0.664	0.025	0.194
PLT (×10⁹/L)	658(230-1200)	930(317-1342)	740(236-1441)	515(43-1576)	0.095	0.488	0.185	0.681	0.005	0.131
Thrombotic event(%)	0	0	0	1.0	–	–	1.0	–	1.0	1.0
Prognosis(n.)					0.090	0.256	<0.001	0.282	<0.001	0.009
Low risk	19	19	10	27	–	–	–	–	–	–
Intermediate risk	12	4	4	48	–	–	–	–	–	–
High risk	0	0	1	26	–	–	–	–	–	–
OS(months)	45(5-82)	38(6-75)	44(8-73)	33(2-168)	1.0	1.0	1.0	1.0	1.0	1.0

ET: essential thrombocythemia; WBC: white blood cell count; Hct: hematocrit; Hb: hemoglobin; PLT: platelets; RBC: red blood cell count; OS: overall survival. P describes differences between different mutant CALR and JAK2 V617F in ET patients' group.

were 44.9% (31 of 69) and 33.3% (23 of 69) in *CALR* mutation positive ET patients (Figure 1C), as well as 25% (1 of 4) and 50% (2 of 4) in *CALR* mutation positive PMF patients (P=0.793 and P=0.888, respectively). There were few other mutation types in the *CALR*-mutated samples (Figure 1C).

ET patients with mutant *CALR* were significantly younger (P<0.001) and had lower white blood cell (WBC) counts (P<0.001), lower hemoglobin (Hb) levels (P=0.002), and higher platelet (PLT) counts (P<0.001) than patients with *JAK2* V617F. No significant difference can be identified between ET patients with mutant *CALR* and *JAK2* V617F in terms of sex and thrombotic events (Table 1). Similarly, PMF patients with mutant *CALR* showed lower Hb level (P=0.001) than *JAK2* V617F. There was no significant difference in sex, age, WBC count, PLT counts or thrombotic events between PMF patients with mutant CALR and *JAK2* V617F (Table 1). For different *CALR* mutations in ET patients, younger age (P=0.020), lower WBC count (P<0.001), and lower Hb level (P=0.002) were observed in *CALR* L367fs*46 than *JAK2* V617F. In addition, ET patients with *CALR* K385fs*47 showed lower age (P<0.001), lower WBC counts (P<0.001), lower Hb levels (P=0.025) and higher PLT counts (P=0.005) than *JAK2* V617F (Table 2).

The overall survival (OS) rates of patients with ET and PMF were analyzed using the Kaplan-Meier curve. Longer OS was observed in ET and PMF patients with mutant *CALR*, but not wt *CALR* (P=0.511 and P=0.729, respectively) (Table 1 and Figure 1D). According to the risk stratification system in ET,[11] there was a significant difference between patients with ET in the *CALR*-mutated group and *JAK2* V617F mutant group or wt *CALR* group (both P<0.001) (Table 1).

In summary, our data from this large cohort of Chinese patients with MPNs confirmed *CALR* mutations were novel molecular markers in wt *JAK2* MPNs. It should always be noted that the combination of *CALR*, *JAK2*, and MPL W515L/K mutation analysis could contribute to the diagnosis of MPNs.[5-7] Different *CALR* mutations of patients with MPNs had distinct clinical characteristics. Patients with the L367fs*46 and K385fs*47 mutations have shown a favorable prognosis, but further research is required to confirm this result. Given the relative proportion of MPN patients without *JAK2/MPL/CALR* mutations in our patient group, further investigation should be carried out to find novel molecular aberrations.

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