

Clinical Manifestation of Calreticulin Gene Mutations in Essential Thrombocythemia without Janus Kinase 2 and MPL Mutations: A Chinese Cohort Clinical Study

Chao Sun¹, Xin Zhou¹, Zhi-Jian Zou¹, Hong-Feng Guo¹, Jian-Yong Li², Chun Qiao²

¹Department of Hematology, Affiliated Wuxi Peoples Hospital, Nanjing Medical University, Wuxi, Jiangsu 214023, China

²Department of Hematology, The First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing, Jiangsu 210029, China

Abstract

Background: Recently, calreticulin (*CALR*) gene mutations have been identified in patients with essential thrombocythemia (ET). A high-frequency of ET cases without Janus kinase 2 (*JAK2*) mutations contain *CALR* mutations and exhibit clinical characteristics different from those with mutant *JAK2*. Thus, we investigated the frequency and clinical features of Chinese patients of Han ethnicity with *CALR* mutations in ET.

Methods: We recruited 310 Chinese patients of Han ethnicity with ET to analyze states of *CALR*, *JAK2V617F*, and *MPLW515* mutations by polymerase chain reaction and direct sequencing. We analyzed the relationship between the mutations and clinical features.

Results: *CALR*, *JAK2V617F*, and *MPLW515* mutations were detected in 30% ($n = 92$), 48% ($n = 149$), and 1% ($n = 4$) of patients with ET, respectively. The mutation types of *CALR* involved deletion and insertion of base pairs. Most of them were Type 1 (52-bp deletion) and Type 2 (5-bp insertion, TTGTC) mutations, leading to del367fs46 and ins385fs47, respectively. The three mutations were exclusive. Clinically, patients with mutated *CALR* had a lower hemoglobin level, lower white blood cell (WBC) count, and higher platelet count compared to those with mutated *JAK2* ($P < 0.05$). Furthermore, a significant difference was found in WBCs between wild-type patients (triple negative for *JAK2*, *MPL*, and *CALR* mutations) and patients with *JAK2* mutations. Patients with *CALR* mutations predominantly clustered into low or intermediate groups according to the International Prognostic Score of thrombosis for ET ($P < 0.05$).

Conclusions: *CALR* mutations were frequent in Chinese patients with ET, especially in those without *JAK2* or *MPL* mutations. Compared with *JAK2* mutant ET, *CALR* mutant ET showed a different clinical manifestation and an unfavorable prognosis. Thus, *CALR* is a potentially valuable diagnostic marker and therapeutic target in ET.

Key words: Calreticulin; Essential Thrombocythemia; Gene Mutation; Janus Kinase 2

INTRODUCTION

According to the 2008 World Health Organization (WHO) classification of hematopoietic tumors, essential thrombocythemia (ET) belongs to *BCR-ABL1* negative myeloproliferative neoplasms (MPNs). The disease is characterized by stem cell-derived clonal myeloproliferation. A diagnosis requires meeting all the following criteria:^[1] platelet (PLT) count $\geq 450 \times 10^9/L$; megakaryocyte proliferation with large and mature morphology; not meeting the WHO criteria for chronic myeloid leukemia, polycythemia vera, primary myelofibrosis, myelodysplastic syndromes, or other myeloid neoplasm; demonstration of *JAK2V617F* or other clonal markers or no evidence of

reactive thrombocytosis. Therefore, *JAK2V617F* and *MPL* mutations have been characteristic molecular markers in ET since 2005 in addition to clinical features and hemogram and marrow picture, whose frequency is 50–60% and 5–10%, respectively. The above mutations explain the pathogenesis

Address for correspondence: Dr. Jian-Yong Li,
Department of Hematology, The First Affiliated Hospital of Nanjing
Medical University, Jiangsu Province Hospital, Nanjing,
Jiangsu 210029 China
E-Mail: lijianyonglm@medmail.com.cn

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of ET from the Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway and provide a theoretical foundation for targeted therapy.^[2-4] However, about one-third of ET cases do not harbor these mutations. Although some other gene aberrations such as mutations in *TET2*, *EZH2*, *DNMT3A*, and *ASXL1* have been detected and are associated with different cellular targets and epigenetic regulatory pathways, they are nonspecific and casual.^[5-7] Fortunately, a new mutation in calreticulin (*CALR*) exon 9 was recently detected in MPNs (especially in ET without *JAK2* and *MPL* mutations) through exome sequencing technology. This novel somatic mutation is associated with clinical features and prognoses of ET and might provide useful leads regarding tumorigenesis.^[8,9] However, few studies have been done on ET patients of Chinese descent. In this study, we analyzed the status of the above mutations and their clinical and laboratory features in a large ($n = 310$) cohort of Chinese patients with ET.

METHODS

Subjects and inclusion criteria

Three-hundred and ten patients with ET were recruited from the Department of Hematology at Wuxi People's Hospital and Jiangsu Province Hospital between January 2007 and June 2015. All patients were diagnosed according to the WHO 2008 criteria for ET classification. We also recruited twenty healthy controls. Every patient provided informed consent for sample collection in accordance with the *Declaration of Helsinki*. The study was approved by the Ethics Committee of both hospitals.

DNA extraction and Janus kinase 2, *MPL*, and calreticulin mutation analyses

Mononuclear cells were extracted from peripheral blood and/or bone marrow of ET patients before treatment by Ficoll–Hypaque gradient centrifugation and cryopreserved on the day of sample collection. Genomic DNA was extracted from 5 to 10×10^6 mononuclear cells using the DNAzol kit (Invitrogen) (Norway Dynal Company) according to the manufacturer's instructions. Polymerase chain reaction (PCR) and clone sequencing were performed for detection of *JAK2V617F* and *MPL* exon 10 mutations as previously described.^[10] Mutations in *CALR* exon 10 were also assessed by PCR followed by direct sequencing. A pair of primers was used to amplify a 306-bp product: forward 5'-ACAACCTTCCTCATCACCAACG-3' and reverse 5'-GGCCTCAGTCCAGCCCTG-3'. The *CALR* PCR conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s; and finally, 72°C for 7 min. The mutations were confirmed by at least two repeated analyses in this study.

Clinical data collection

The following clinical data were collected and analyzed: age, sex, peripheral blood count, event of major thrombosis, and International Prognostic Score for ET-Thrombosis system (IPSET-T).

Statistical analyses

Statistical analyses were performed with the statistical software GraphPad Prism 5.0 (GraphPad Software, USA). Discrete variables such as sex, event of major thrombosis, and IPSET-T were analyzed by the Chi-square test between different mutation groups and the wild-type group. Continuous variables including age and peripheral blood count were summarized by median and range and analyzed using the Mann–Whitney test. Overall survival (OS) analysis was considered from the date of diagnosis to the date of death (uncensored) or last contact (censored) and prepared by the Kaplan–Meier method. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Frequency and pattern of the calreticulin mutation

Among the 310 ET cases, *CALR* mutations were identified in 92 (30%), *JAK2V617F* in 149 (48%), and *MPLW515* in 4 (1%) cases, whereas 65 (21%) cases were negative for all three mutations (wild-type) [Figure 1a]. All four *MPL*-mutated cases were the *MPLW515* mutation. The three mutations were exclusive in ET. We did not observe the *CALR* mutation or the other two mutations in healthy controls. In patients without *JAK2V617F* and *MPL* (wild-type), the frequency of *CALR* mutations was much higher at 59% (92/157). All 11 *CALR* mutation types were detected in our ET cases, including nucleotide deletion or insertion. The most common mutation was Type 1 (c.1092_1143del 52-bp; 50%, 45/92) leading to L367fs*46, followed by Type 2 (c.1154_1155insTTGTC; 35%, 32/92) leading to K385fs*47 [Figures 1b and 2a].

Relation between clinical phenotype and different mutations

We divided all cases into three subgroups according to the different mutation types (*MPL*-mutated cases were eliminated because of the small sample size): *CALR*-mutated, *JAK2V617F*, and wild-type groups. Table 1 shows the clinical and laboratory characteristics of the 306 ET cases. As shown in Table 1, cases with mutated *CALR* had a lower hemoglobin (Hb) level, lower white blood cell (WBC) count, and higher PLT count, as well as a higher chance of being younger than those with mutated *JAK2* ($P < 0.05$). No significant differences were observed in Hb level, PLT count, or age between the *CALR*-mutated and wild-type groups; however, a significant difference was found for WBC count. Similar clinical features were observed between the Type 1 or Type 2 groups and the *JAK2V617F* group, and there was no significant difference in clinical and laboratory features between the Type 1 and Type 2 *CALR* mutation groups [Table 2].

Thrombotic event, overall survival, and International Prognostic Score for Essential Thrombocythemia-Thrombosis between different mutations

According to our data [Table 1 and Figure 3], there was no significant difference in thrombotic events and OS among

Table 1: Clinical features of ET patients with *CALR* and *JAK2* mutation

Characteristics	<i>CALR</i>	<i>JAK2V617F</i>	Wild-type	<i>P</i>	
				<i>CALR</i> versus <i>JAK2V617F</i>	<i>CALR</i> versus wild-type
Number	92	149	65		
Sex (male:female)	44:48	73:76	33:32	0.89	0.74
Age (years)	55 (20–85)	62 (23–95)	45 (18–80)	<0.001	0.08
WBC ($\times 10^9/L$)	8.2 (5.8–12.9)	15.6 (4.5–111.3)	9.5 (3.2–33.7)	<0.001	<0.001
Hb (g/L)	126 (100–167)	149 (76–212)	121.5 (79–221)	0.002	0.36
PLT ($\times 10^9/L$)	996 (465–1941)	601 (460–1602)	629 (567–1881)	<0.001	0.26
Thrombotic event (%)	1	2	4.6	1.0	0.3075
IPSET-T (<i>n</i>)				<0.001	<0.001
Low-risk	58	25	25	–	–
Intermediate risk	30	68	19	–	–
High-risk	4	56	21	–	–

ET: Essential thrombocythemia; IPSET-T: International Prognostic Score of thrombosis in World Health Organization–essential thrombocythemia; WBC: White blood cell; PLT: Platelet; Hb: Hemoglobin; *JAK2*: Janus kinase 2; *CALR*: Calreticulin; –: Not available.

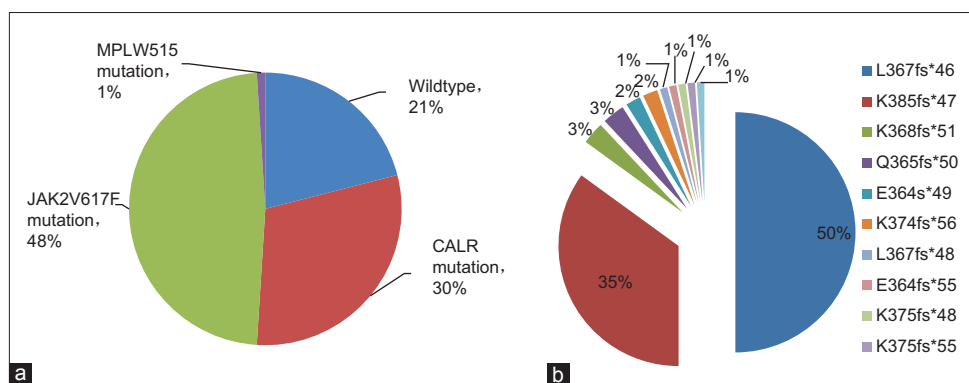


Figure 1: (a) Distribution of Janus kinase 2, *MPL*, and calreticulin mutations in essential thrombocythemia. (b) Different mutation types in essential thrombocythemia with calreticulin mutation.

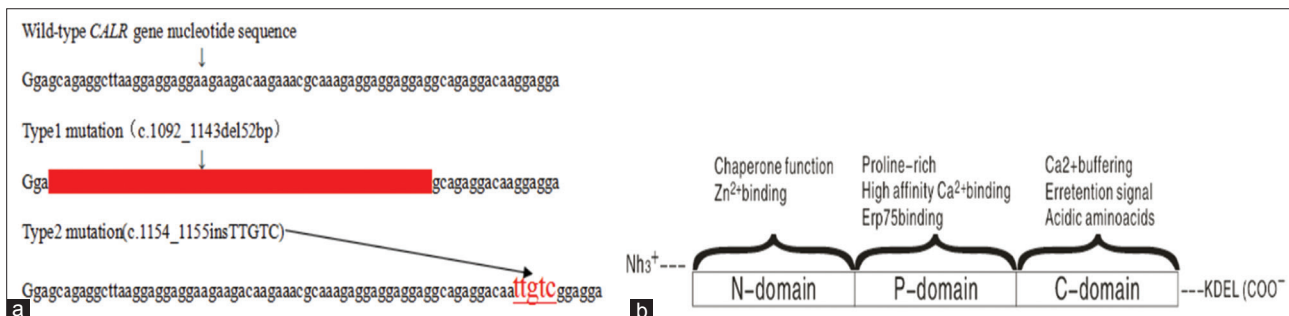


Figure 2: (a) Comparison between wild-type and mutant calreticulin gene sequences (deletion and insertion nucleotide in red). (b) Structure and function of calreticulin protein.

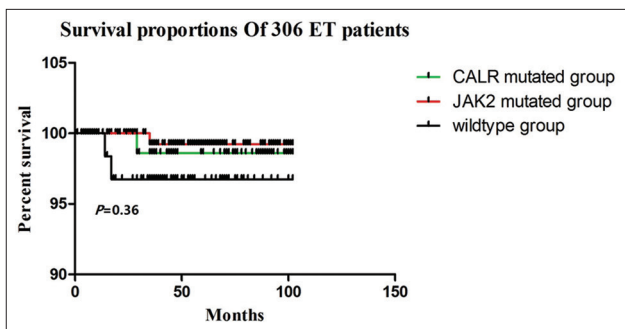


Figure 3: Kaplan–Meier estimates of overall survival in 306 patients with essential thrombocythemia stratified by mutation type.

the three groups. According to the IPSET-T stratification, a close relationship of the *CALR* mutations to the low- or intermediate-risk group was observed ($P < 0.05$).

DISCUSSION

ET belongs to MPN and is characterized by thrombocytosis and other features including leukocytosis, splenomegaly, thrombosis, bleeding, microcirculatory symptoms, pruritus, and risk of leukemic or fibrotic transformation. The median survival is 20 years for ET, and the leukemic transformation rate at 20 years is estimated to be <5%.^[11,12] Thrombosis and bleeding are the major causes of death.^[13,14] Identification of

Table 2: Clinical features of ET patients with Types 1 or 2 of *CALR* and *JAK2* mutation

Characteristics	Type 1	Type 2	<i>JAK2V617F</i>	P		
				Type 1 versus Type 2	Type 1 versus <i>JAK2V617F</i>	Type 2 versus <i>JAK2V617F</i>
Number (male/female)	45 (22:23)	32 (17:15)	149 (73:76)	0.81	1	0.7008
Age (years)	52 (20–78)	42 (22–80)	62 (23–95)	0.065	0.020	<0.001
WBC ($\times 10^9/L$)	7.9 (5.8–12.9)	6.8 (6.1–10.9)	15.6 (4.5–111.3)	0.159	<0.001	<0.001
Hb (g/L)	119 (100–167)	122 (102–157)	149 (76–212)	0.671	0.002	0.025
PLT ($\times 10^9/L$)	768 (465–1941)	890 (750–1342)	601 (460–1602)	0.095	0.185	0.005
Thrombotic event (%)	0	0	2	–	1.0	1.0
IPSET-T (n)				0.246	<0.001	<0.001
Low-risk	26	24	25	–	–	–
Intermediate risk	18	7	68	–	–	–
High-risk	1	1	56	–	–	–

ET: Essential thrombocythemia; IPSET-T: International Prognostic Score of thrombosis in World Health Organization–essential thrombocythemia; WBC: White blood cell; PLT: Platelet; Hb: Hemoglobin; *JAK2*: Janus kinase 2; *CALR*: Calreticulin; –: Not available

JAK2V617F and *MPL* mutations has been applied widely for accurate diagnosis of about 50–60% cases of ET since 2005, whereas *CALR* mutations seem to play an important role in cases without *JAK2V617F* and *MPL* mutations as observed since 2013.^[8,9]

CALR is located on chromosome 19p13.2 and is 5891-bp long with nine exons. The encoded protein has 417 amino acids and consists of three main structural and functional domains [Figure 2b]. As a protein with different functions,^[15] *CALR* exists both in the endoplasmic reticulum (ER) and outside the ER including on the cell surface and in the cytoplasm, nucleus, and extracellular matrix. Within the ER, the main physiological function of the protein includes chaperone activity and modulation of calcium homeostasis; outside the ER, it is associated with the healing of cutaneous wounds, immunogenic response, and regulation of cell adhesion, translation, gene expression, and nuclear export.^[16–18] Thus, *CALR* is crucial in cellular activity, and its dysfunction is associated with the disruption of normal cellular physiological and pathological processes, which could lead to oncogenic consequences.^[19] Owing to frameshift mutations, proteins encoded by mutated *CALR* harbor a loss of the C-terminal KDEL domain (Lys-Asp-Glu-Leu), generating a novel peptide sequence terminating with the same 36 amino acids. This alteration transforms the negatively charged glutamic acid-rich C-terminus of wild-type *CALR* to a positively charged arginine-rich region.^[20,21] Therefore, the mutant protein may influence Ca^{2+} binding and normal cell growth. Using retroviral production and transfection of Type-1 *CALR*-mutated cDNAs into the Ba/F3 cell line, interleukin-3-independent growth, and STAT5 activation have been observed. This suggests that mutant *CALR* proteins may disrupt cell growth via the STAT pathway.^[8]

The total prevalence of *CALR* mutations in ET is 15–30%, whereas in ET without *JAK2* and *MPL* mutations, *CALR* mutation frequency ranges from 50% to 70%. The mutations are mutually exclusive of *JAK2* and *MPL* mutations in ET. The mutation type of *CALR* involves deletion and insertion

of a certain number of base pairs, leading to a shift in the reading frame. Most of them are Type 1 (52-bp deletion) and Type 2 (5-bp insertion, TTGTC) mutations, leading to del367fs46 and ins385fs47, respectively. Other complex mutations such as deletion-merging insertion, which might cause a constitutive change, can also be detected.^[8,9] In our study, the total frequency and mutational hotspots were consistent with western countries. However, the frequency of Chinese patients without *JAK2* and *MPL* mutations seemed to be low when compared to that of patients in England (71.4% 80/112; $P < 0.05$) and complex mutations were not detected.^[9] We attribute this to two possible reasons: the different samples or characteristics of the Chinese population.

Clinically, like in previous studies,^[22] our *CALR*-mutated ET patients presented with younger age, lower Hb level, lower WBC count, and higher PLT count compared to patients with the *JAK2* mutation. The two major *CALR* mutant types also displayed the same features compared to patients with the *JAK2* mutation. No significant differences were observed in peripheral blood count, age, or sex between the Type 1 and Type 2 mutation groups. This showed that although there were various forms of *CALR* mutations, their clinical laboratory parameters were similar, which was in contrast to observations in patients with the *JAK2* mutation. In addition, patients with *CALR* mutations had lower a lower risk of a thrombosis, according to our data, although a statistical difference was not observed, whereas some foreign research data have been documented.^[23] Therefore, granulocyte/PLT activation and increased WBC counts may be more relevant to the pathogenesis of thrombotic complications than absolute PLT counts.

In terms of ET prognosis, the following three aspects are currently evaluated: OS, acute myeloid leukemia (AML) evolution, and thrombosis-free survival (TFS). The earlier OS models considered three risk factors – age (≥ 60 years), WBC count ($> 15 \times 10^9/L$ or $11 \times 10^9/L$), and anemia – and categorized patients into low-risk (no risk factor), medium-risk (1 risk factor), and high-risk groups (≥ 2 risk factors). AML evolution categorizes patients into these

groups based on $PLT > 1000 \times 10^9/L$ and anemia: low-risk (no risk factor), medium-risk (1 risk factor), and high-risk groups (2 risk factors).^[24-26] In 2012, Passamonti *et al.* proposed the IPSET system based on analysis of 867 cases of WHO-diagnosed ET. This model includes three parameters: age (2 points for ≥ 60 years) while blood cell count (1 point for $\geq 11 \times 10^9/L$) and previous thrombotic events (1 point). Patients were then categorized into low-risk (sum = 0; median survival not reached), medium-risk (sum = 1–2; median survival 24.5 years), and high-risk (sum = 3–4; median survival 13.8 years) groups based on overall scores to evaluate different OS of patients in different risk stratifications. This model is both suitable for the OS evaluation of ET corresponding to the WHO diagnostic standard and those that do not meet the WHO diagnostic standard but have suitable clinical presentations, as well as TFS prognosis evaluation.^[27]

TFS prognosis evaluation in ET patients previously considered two factors—age (≥ 60 years) and previous thrombotic events—to categorize patients into low-risk (no risk factor) and high-risk groups (more than 1 risk factor).^[28] Along with evidence that *JAK2* mutations and cardiovascular risk factors are closely linked to thrombotic events,^[29,30] a new thrombosis prognosis model, IPSET-T was established. This model included *JAK2* mutations (2 points), cardiovascular risk factors (1 point for tobacco use, hypertension, or diabetes mellitus), age (1 point for ≥ 60 years), and previous thrombotic events (2 points) and categorized patients into low-, medium-, and high-risk groups. Based on this model, almost 50% of low-risk patients were re-grouped into the new medium-risk (47%) or high-risk groups (5%), while nearly two-thirds of the previously high-risk patients were now in the low- or medium-risk groups (one-third each). This categorization resulted in three groups of patients with distinctly different TFSs and presented the different thrombosis event risks of patients who have different clinical and biological characteristics.^[31] According to our short follow-up period (median follow-up time was 102 months), no difference was observed between different mutation groups on OS. However, current reports about OS are not consistent. Klampfl *et al.* show that patients with *CALR* mutations had better OS than those with *JAK2* mutations, whereas no difference was observed according to Nangalia *et al.* and Rotunno *et al.*^[8,9,20] Recently, the long-term follow-up study of 266 ET cases by Tefferi *et al.* showed no difference in the OS of patients with *CALR* and *JAK2* mutations even when analyzed in different age groups. In addition, the two groups of patients showed no significant difference in leukemia prognosis and myelofibrosis.^[32] In comparison, Palandri *et al.* found from long-term follow-up of 217 ET patients who were < 40 years old that those with *CALR* mutations had better OS and TFS than those with *JAK2* mutations.^[33] Thus, the effect of *CALR* mutations on the OS of ET patients requires further clarification with larger sample sizes and longer follow-up time in multi-race clinical studies. Although OS of our data had no statistical difference, the patients were divided into low-, medium-,

and high-risk groups based on IPSET-T, and the different mutation distributions were compared in these groups. *CALR* mutations mainly distributed in the medium- and low-risk groups while *JAK2* mutations showed a focused distribution in the medium- and high-risk groups. This could be indirect evidence of the effect of *CALR* mutations on prognosis. However, a recent study found no independent effect of *CALR* mutations on prognosis in a multivariate analysis of TFS and had no substantial guiding significance to the IPSET-T model.^[34] Further clarification from additional studies with larger sample sizes is needed.

In summary, *CALR* mutations are novel somatic mutations in ET. This finding will assist with understanding ET that does not involve *JAK2* and *MPL* mutations. Compared to ET with *JAK2* mutations, ET with *CALR* mutations in Chinese patients showed a different clinical manifestation and different prognosis, which is similar to results from western countries. *CALR* might be a valuable diagnostic marker and therapeutic target in ET^[35] leading to the development of innovative diagnostic criteria and therapies for MPN patients.

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Conflicts of interest

There are no conflicts of interest.

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