Frequencies, Laboratory Features, and Granulocyte Activation in Chinese Patients with CALR-Mutated Myeloproliferative Neoplasms

Haixiu Guo¹²*, Xiuhua Chen¹*, Ruiyuan Tian¹, Jianmei Chang¹, Jianlan Li¹, Yanhong Tan¹, Zhifang Xu¹, Fanggang Ren¹, Junxia Zhao¹, Jie Pan¹, Na Zhang¹, Xiaojuan Wang¹, Jianxia He¹, Wanfang Yang¹, Hongwei Wang¹*

¹ Institute of Hematology, the Second Hospital of Shanxi Medical University, Taiyuan, China, ² Department of Microbiology and Immunology, School of Basic Medicine, Shanxi Medical University, Taiyuan, China

☯ These authors contributed equally to this work.
* wanghw68@hotmail.com

Abstract

Somatic mutations in the CALR gene have been recently identified as acquired alterations in myeloproliferative neoplasms (MPNs). In this study, we evaluated mutation frequencies, laboratory features, and granulocyte activation in Chinese patients with MPNs. A combination of qualitative allele-specific polymerase chain reaction and Sanger sequencing was used to detect three driver mutations (i.e., CALR, JAK2V617F, and MPL). CALR mutations were identified in 8.4% of cases with essential thrombocythemia (ET) and 5.3% of cases with primary myelofibrosis (PMF). Moreover, 25% of polycythemia vera, 29.5% of ET, and 48.1% of PMF were negative for all three mutations (JAK2V617F, MPL, and CALR). Compared with those patients with JAK2V617F mutation, CALR-mutated ET patients displayed unique hematological phenotypes, including higher platelet counts, and lower leukocyte counts and hemoglobin levels. Significant differences were not found between Chinese PMF patients with mutants CALR and JAK2V617F in terms of laboratory features. Interestingly, patients with CALR mutations showed markedly decreased levels of leukocyte alkaline phosphatase (LAP) expression, whereas those with JAK2V617F mutation presented with elevated levels. Overall, a lower mutant rate of CALR gene and a higher triple-negative rate were identified in the cohort of Chinese patients with MPNs. This result indicates that an undiscovered mutant gene may have a significant role in these patients. Moreover, these pathological features further imply that the disease biology varies considerably between mutants CALR and JAK2V617F.
Introduction
The three classic BCR-ABL-negative myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). In early 2005, a unique gain-of-function mutation in the Janus kinase 2 gene (JAK2V617F) was identified in approximately 95% of patients with PV and in 50% to 60% of patients with ET or PMF [1,2]. Subsequently, somatic mutations of JAK2 exon 12 are found in most PV patients without JAK2V617F mutation [3,4], whereas the thrombopoietin receptor (MPL) mutations are present in a small number of patients with ET or PMF [5,6]. The discovery of these mutations has greatly facilitated the diagnosis of the three disorders. However, approximately one-third of patients with ET or PMF do not carry JAK2 or MPL mutation, and the molecular basis of these neoplasms remains unknown.

Somatic mutations in the calreticulin (CALR) gene were detected in about 25% to 35% of all patients with ET and PMF [7,8]. These mutations are mutually exclusive with mutations in both JAK2 and MPL. More than 50 different types of mutations in CALR have been detected to date, but the 52 bp deletion (type 1 mutation, c.1179_1230del) and the 5 bp insertion (type 2 mutation, c.1234_1235insTTGTC) are the most frequent. Overall, these mutations are found in 80% to 90% of all tested patients with mutant CALR. All reported CALR mutations result in a frameshift, owing to insertions or deletions in the last exon (exon 9). Functional analysis showed that overexpression of the most frequent CALR deletion can cause cytokine-independent growth in vitro and result in the activation of STAT5 through an unknown mechanism [7,8]. These data suggest that CALR mutations play a causal role during the development and evolution of MPNs, similar to JAK2 and MPL mutations.

In this study, we investigated the profile and laboratory features of CALR mutations in Chinese patients with MPN. We also compared the differences between the present findings and those from four different geographical sites in China and Western countries. To further explore the biological effects of CALR mutations, we analyzed the leukocyte alkaline phosphatase (LAP) characteristics of the MPN patients with CALR mutations. This study is the first to report the relationship between CALR mutations and LAP expression in MPN patients.

Materials and Methods
Ethics statement
This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of Shanxi Medical University. Written informed consent was obtained from all patients and from the legal guardians in the case of minors.

Patient samples
A total of 668 MPN patients, including 128 PV, 407 ET, and 133 PMF, were diagnosed according to the World Health Organization criteria [9]. The relevant diagnosis criteria for JAK2 mutation-negative PV included bone marrow histology, serum Epo levels, and endogenous erythroid colonies (EECs) [9]. The patients were recruited from the Department of Hematology, the Secondary Hospital of Shanxi Medical University, Shanxi Province, China. These patients had stored samples of granulocyte DNA from bone marrow or peripheral samples. The patients’ laboratory features, including age, gender, and hematological parameters, were obtained from the medical records at diagnosis. LAP expression was evaluated by LAP score. The patients’ LAP scores were obtained from the medical records at diagnosis and were tested through routine clinical testing conducted in our laboratory. LAP scores were measured using the LAP staining kit (Sunny Biotechnology, Shanghai, China) according to the manufacturer’s
instructions. The procedure involves counting 100 neutrophils, including lobed and band forms, but excluding other left-shifted granulocytes, eosinophils, and basophils. The reaction was scored from 0 to 4 depending on the number of stained granules and the intensity of the stain. The number of cells was multiplied by the score and added up to a normal range (40–80). Puerperal is applied to positive contrast.

**JAK2V617F, MPL, and CALR mutation assays**

Allele-specific PCR (AS-PCR) was introduced for the screening of JAK2V617F point mutation. The reverse primer (V617F primer: 5’-GTGTTACTCTACTCTCGTCTCCACAAAA-3’) of AS-PCR was specific for the mutant allele and contains an intentional mismatch at the last nucleotide from the 3’ end. The outer primer pairs (forward primer: 5’-CCTCAGAAGTTGATGCA-3’, reverse primer: 5’-ATGTGCTCTTCTTTTCTACAAGA-3’) were designed to amplify fragments from the V617F mutant and wild-type alleles as internal control. The PCR product was analyzed using an ethidium bromide-impregnated 2% agarose gel electrophoresis. The electrophoresis result showed a 295 bp mutant-type band and a 451 bp wild-type band.

Genomic PCR amplification combined with directional Sanger sequencing was applied to screen CALR and MPL mutations. A pair of oligonucleotide primers covering exon 9 of CALR was used to amplify a 1019 bp product (forward: 5’-AAACCCTTGCTCAAAGCAAG-3’ and reverse: 5’-GGAGACACAAAATTTAATTTAATAG-3’). Oligonucleotide primers targeting MPL exon 10 were employed to amplify a 218 bp product (forward: 5’-TAGGGGCTGGGTAG-3’ and reverse: 5’-CTTCGGCTCCACCTGGTCC-3’). PCR products were purified and subjected to directional sequencing.

**Statistical analysis**

Statistical analyses were performed using SPSS 13.0 software. Numerical variables were summarized by median and range, and categorical variables by count and relative frequency (%) of each category. The parameters between patient groups were statistically analyzed with the nonparametric Wilcoxon rank-sum test (for measurement data) and the Fisher’s exact test or Pearson Chi-square test (for enumeration data). *P* < 0.05 was considered statistically significant (two-tailed).

**Results**

**Mutational frequencies of CALR gene in patients with MPN**

Three mutations, namely, JAK2V617F, MPL, and CALR, were simultaneously screened in a cohort of 668 Chinese patients with confirmed PV (N = 128), ET (N = 407), and PMF (N = 133) from our center to estimate CALR mutation profile in Chinese patients with MPN. As shown in Table 1, JAK2V617F mutation was detected in 75.0% of PV, 61.2% of ET, and 45.1% of PMF. MPL exon 10 mutations were identified in six patients. MPLW515L was found in two cases with ET and one case with PMF; MPLW515K was detected in one case with PMF; and MPLA497-L498LVIALins was found in two cases with ET. MPLA497-L498LVIALins is an insertion mutation previously reported by our group [10]. CALR mutations were identified in 34 patients with ET and 7 with PMF, accounting for 8.4% of cases with ET and 5.3% of cases with PMF, respectively. In our cohort, none of the patients with PV were found harboring MPL and CALR mutations. The three mutations (JAK2V617F, MPL, and CALR) were mutually exclusive in patients with ET and PMF. Notably, 29.5% of ET and 48.1% of PMF patients showed no mutations on all three genes (triple-negative).

For mutation types, 12 distinct variants of CALR mutations were identified in our cohort, including 6 deletions (c.1179_1230del, c.1174_1219del, c.1170_1221del, c.1185_1218del, c.1196_
1226del, and c.1179_1221del), 4 insertions (c.1234_1235insTTGTC, c.1235_1236insTGGC, c.1237_1238insAAAG, and c.1228_1229insTCTTTGTC), and 2 complex indels (c.1179_1233delinsAGG and c.1200_1220delinsTCTTTGTC). Among these mutation types, c.1179_1230del (mutation type 1, a 52 bp deletion) and c.1234_1235insTTGTC (mutation type 2, a 5 bp insertion) were the most frequent alterations, representing 51.2% and 24.4% of cases with mutant CALR, respectively. The remaining 10 types were previously unidentified, novel CALR mutations. Consistent with the previously described CALR mutations, all novel mutant variants caused a +1 bp frameshift and led to a novel C-terminal peptide sequence. These novel mutations were only observed in a single ET patient. In ET patients with mutant CALR, type 1 mutations were the most common (41.2%), followed by type 2 (26.5%), and the other 10 novel mutation types were found in one ET patient (2.9%). In PMF patients with mutant CALR, only types 1 (85.7%) and 2 (14.3%) were detected (Table 2).

Demographic and laboratory features of ET or PMF patients stratified by CALR and JAK2V617F mutations

Medical records at diagnosis of Chinese patients with CALR or JAK2V617F mutations were collected to define their demographic and laboratory features. Only 267 patients with ET and 62 patients with PMF had complete records. Among patients with ET, those with CALR
mutations (CALR+) showed lower hemoglobin (Hb) levels (P < 0.001), lower white blood cell (WBC) counts (P < 0.001), and higher platelet (PLT) counts (P = 0.003) than those with JAK2V617F mutation (JAK2V617F+) (Table 3). Frequencies for age and gender were balanced between the two groups (Table 3). For different CALR mutation types in ET patients, statistical difference was not observed in age, gender, platelet counts, Hb levels, and WBC counts between CALR type1+ group and CALR type2+ group (Table 4). Compared with patients with JAK2V617F+ (Table 4), patients with CALR type1+ presented lower WBC counts (P = 0.003). Male patients with CALR type1+ showed higher Hb levels (P = 0.025) than those with JAK2V617F+, whereas female patients with CALR type1+ showed lower Hb levels (P = 0.000) than ones with JAK2V617F+. No statistical differences were observed for age, gender, platelet counts between patients with CALR type1+ and JAK2V617F+. Patients with CALR type2+ exhibited lower WBC counts (P = 0.002) and higher platelet counts (P = 0.002) than patients with JAK2V617F+ (Table 4). No statistical differences were showed for Hb levels between male patients with CALR type2+ and JAK2V617F+, whereas female patients with CALR type2+ presented lower Hb levels (P = 0.003) than female patients with JAK2V617F+. Significant differences were not found in age and gender between patients with CALR type2+ and JAK2V617F+. Similarly, CALR+ and JAK2V617F+ shared similar laboratory features in patients with PMF. These groups showed no significant differences in age, gender, Hb levels, and WBC and PLT counts (Table 3). Among PMF patients with different CALR mutation types, only one patient with CALR type2+ had a complete record. Hence, comparison analysis was only conducted in CALR type1+ and JAK2V617F+ patients. No significant differences were noted in age, gender, Hb levels, and WBC and PLT counts between CALR type1+ and JAK2V617F+ (Table 3).

**Table 2. Comparison analysis of MPN phenotypes between types 1 and 2 mutations in CALR gene.**

| Different regions of China | Western countries |
|---------------------------|-------------------|
| **ET cohort**             |                   |
| Type 1                    | 41.2% (14/34)     |
| Type 2                    | 26.5% (9/34)      |
| **PMF cohort**            |                   |
| Type 1                    | 85.7% (6/7)       |
| Type 2                    | 14.3% (1/7)       |

**Table 3. Demographic and laboratory features at diagnosis of ET and PMF patients with CALR and JAK2V617F mutations.**

|                  | ET cohort | PMF cohort | P value |
|------------------|-----------|------------|---------|
|                  | (A) CALR+ (N = 29) | (B) JAK2V617F+ (N = 238) | (C) CALR+ (N = 6) | (D) JAK2V617F+ (N = 56) | (A) vs (B) | (C) vs (D) |
| Age, median (range)(years) | 59 (15–90) | 58 (23–78) | 63 (53–71) | 60 (22–81) | 0.872 | 0.957 |
| Sex (male:female) | 17:12 | 108:130 | 2:4 | 25:31 | 0.177 | 0.689 |
| WBC count, median (range) (x10^9/L) | 12.2 (4.1–37) | 15.4 (4.1–77.1) | 5.2 (2.6–10.2) | 15.9 (0.5–110.0) | 0.001 | 0.096 |
| Hb, median (range) (g/L) | 123.8 (66.4–172.0) | 145.4 (52.6–428) | 116.9 (70.0–168.0) | 111.3 (47.6–169.0) | 0.001 | 0.710 |
| PLT count, median (range) (x10^9/L) | 1023 (420–2640) | 764 (404–2274) | 204 (115–254) | 177 (18–383) | 0.003 | 0.482 |

WBC: white blood cell; Hb: hemoglobin; PLT: platelet.

Type 1: c.1179_1230del; Type 2: c.1234_1235insTTGTC; “—” indicates no detection performed in the corresponding studies.

**Table 2. Comparison analysis of MPN phenotypes between types 1 and 2 mutations in CALR gene.**

|                  | Our group | Qiao et al. | Chen et al. | Shen et al. | Li et al. | Nangalia et al. | Klampfl et al. |
|------------------|-----------|-------------|-------------|-------------|-----------|----------------|----------------|
| **ET cohort**    |           |             |             |             |           |                |                |
| Type 1           | 41.2% (14/34) | 44.9% (31/69) | 54.5% (18/33) | 46.0% (23/50) | —           | 38.8% (38/98) | 46.2% (90/195) |
| Type 2           | 26.5% (9/34)  | 33.3% (23/69) | 27.3% (9/33)  | 30.0% (15/50) | —           | 48.0% (47/98) | 37.9% (74/195) |
| **PMF cohort**   |           |             |             |             |           |                |                |
| Type 1           | 85.7% (6/7)   | 25.0% (1/4)   | —           | —           | 31.6% (24/76) | 60.5% (23/38) | 65.7% (69/105) |
| Type 2           | 14.3% (1/7)    | 50.0% (2/4)    | —           | —           | 64.5% (49/76) | 23.7% (9/38)  | 20.0% (21/105) |

Type 1: c.1179_1230del; Type 2: c.1234_1235insTTGTC; “—” indicates no detection performed in the corresponding studies.

**Table 3. Demographic and laboratory features at diagnosis of ET and PMF patients with CALR and JAK2V617F mutations.**

|                  | ET cohort | PMF cohort | P value |
|------------------|-----------|------------|---------|
|                  | (A) CALR+ (N = 29) | (B) JAK2V617F+ (N = 238) | (C) CALR+ (N = 6) | (D) JAK2V617F+ (N = 56) | (A) vs (B) | (C) vs (D) |
| Age, median (range)(years) | 59 (15–90) | 58 (23–78) | 63 (53–71) | 60 (22–81) | 0.872 | 0.957 |
| Sex (male:female) | 17:12 | 108:130 | 2:4 | 25:31 | 0.177 | 0.689 |
| WBC count, median (range) (x10^9/L) | 12.2 (4.1–37) | 15.4 (4.1–77.1) | 5.2 (2.6–10.2) | 15.9 (0.5–110.0) | 0.001 | 0.096 |
| Hb, median (range) (g/L) | 123.8 (66.4–172.0) | 145.4 (52.6–428) | 116.9 (70.0–168.0) | 111.3 (47.6–169.0) | 0.001 | 0.710 |
| PLT count, median (range) (x10^9/L) | 1023 (420–2640) | 764 (404–2274) | 204 (115–254) | 177 (18–383) | 0.003 | 0.482 |

WBC: white blood cell; Hb: hemoglobin; PLT: platelet.

doi:10.1371/journal.pone.0138250.t002
doi:10.1371/journal.pone.0138250.t003
JAK2V617F+ patients (Table 5). Laboratory features of the PMF patient with CALR type2+ are described in Table 5.

Analysis of LAP expression in PV, ET, and PMF patients with CALR and JAK2V617F mutations

LAP scores were evaluated in patients with CALR and JAK2V617F mutations to assess their effect on granulocyte function. LAP is a key marker for granulocyte activation. High levels of LAP indicate elevated granulocyte activation. LAP scores were obtained through routine clinical testing conducted in our laboratory; the normal reference value of LAP scores ranges from 40 to 80. LAP scores of 19 patients with CALR mutations (ET = 18, PMF = 1) and 136 patients with JAK2V617F mutation (PV = 38, ET = 74, PMF = 24) were obtained at diagnosis. During the diagnosis, none of the patients presented with fever or inflammation, or received previous cytoreductive treatment. LAP score features of the two mutations are shown in Fig 1. In the JAK2V617F-mutated group, all 136 patients presented higher LAP scores than the normal upper limit (>80). Furthermore, the distribution of LAP scores was investigated among patients with PV, ET, and PMF. Patients with PV presented the highest LAP level (median: 168; range: 108–283), followed by patients with PMF (median: 148; range: 83–242) and patients with ET (median: 142; range: 81–353). Thus, the Kruskal–Wallis test demonstrated significant

Table 4. Demographic and laboratory features at diagnosis of ET patients with different types of CALR and JAK2V617F mutations.

|                          | ET cohort | P value |
|--------------------------|-----------|---------|
|                          | (A) CALR-type1*(N = 14) | (B) CALR-type2*(N = 8) | (C) JAK2V617F*(N = 238) |
| Age, median (range) (years) | 55 (24–72) | 57 (31–78) | 58 (23–78) | P = 0.804 | P = 0.515 | P = 0.858 |
| Sex (male:female)         | 9:5       | 5:3     | 108:130   | P = 1.000 | P = 0.168 | P = 0.476 |
| WBC count, median (range) (×10⁹/L) | 10.7 (4.4–37.0) | 8.4 (6.7–12.2) | 15.4 (4.1–77.1) | P = 0.616 | P = 0.003 | P = 0.002 |
| Hb, median (range) (g/L)  | 140.0 (91.3–157.0) | 124.3 (111.0–172.0) | 125.0 (52.6–144.0) | P = 0.548 | P = 0.025 | P = 0.402 |
| PLT count, median (range) (×10⁹/L) | 900 (420–1863) | 1396 (618–2640) | 764 (404–2274) | P = 0.082 | P = 0.078 | P = 0.002 |

WBC: white blood cell; Hb: hemoglobin; PLT: platelet.

Table 5. Demographic and laboratory features at diagnosis of PMF patients with different types of CALR and JAK2V617F mutations.

|                          | PMF cohort | P value |
|--------------------------|------------|---------|
|                          | (A) CALR-type1*(N = 5) | (B) CALR-type2*(N = 1) | (C) JAK2V617F*(N = 56) |
| Age, median (range) (years) | 64 (53–71) | 70 | 60 (22–81) | — | P = 0.841 | — |
| Sex (male:female)         | 2:3 female | female | 25:31 | — | P = 1.000 | — |
| WBC count, median (range) (×10⁹/L) | 4.58 (2.58–10.20) | 3.34 | 15.9 (0.5–110.0) | — | P = 0.103 | — |
| Hb, median (range) (g/L)  | 117.0 (70.0–168.0) | 95.1 | 111.3 (47.6–169.0) | — | P = 0.953 | — |
| PLT count, median (range) (×10⁹/L) | 223 (175–254) | 220 | 177 (18–383) | — | P = 0.441 | — |

WBC: white blood cell; Hb: hemoglobin; PLT: platelet; “—” indicates no analysis performed.
differences in LAP expression among the three groups ($P < 0.001$ for all comparisons). In the CALR-mutated group, the majority of patients with ET or PMF exhibited lower LAP scores. In detail, 16 out of 18 patients with ET and 1 patient with PMF presented significantly lower LAP values than the normal. LAP scores of the other two patients with ET were equal to or slightly

**Fig 1.** (A) LAP scoring images of CALR and JAK2V617F mutant patients. Peripheral blood smear of a representative CALR mutant patient displays a poor LAP expression (positive-cells:1%; score:1). In contrast, peripheral blood smear of a representative JAK2V617F mutant patient reveals a marked LAP expression (positive-cells:90%; score:194). Peripheral blood smear of a representative positive control (Puerperal) shows the highest intensity level of the stain (level: 3). (B) Leukocyte alkaline phosphatase (LAP) scores in circulating granulocytes of peripheral blood from different categories of patients carrying JAK2V617F or CALR mutation. LAP values are shown in a scatter plot; diamond indicates the median. (i) LAP score levels of 38 PV, 74 ET, and 24 PMF patients carrying JAK2V617F mutant alleles. LAP values of all patients are above normal (>80). The Kruskal–Wallis test showed significant differences between the three disorders ($P < 0.001$ for all comparisons). (ii) LAP score levels of 18 ET patients and 1 PMF patient carrying CALR mutant alleles. The entire data showed no significant differences between the ET and PMF groups. Both ET and PMF patients carrying the CALR mutation exhibited lower LAP scores than the normal value. (iii) LAP score levels of 74 ET patients with JAK2V617F mutant alleles and 18 ET patients carrying the CALR mutation. The Mann–Whitney U test showed significant differences between both groups ($P < 0.001$). (iv) LAP score levels of 24 PMF patients with JAK2V617F mutant alleles and 1 PMF patient carrying the CALR mutation. These data show that LAP score levels of PMF patients with JAK2V617F mutation are markedly higher than those with the CALR mutation.

**Fig 1.** (A) LAP scoring images of CALR and JAK2V617F mutant patients. Peripheral blood smear of a representative CALR mutant patient displays a poor LAP expression (positive-cells:1%; score:1). In contrast, peripheral blood smear of a representative JAK2V617F mutant patient reveals a marked LAP expression (positive-cells:90%; score:194). Peripheral blood smear of a representative positive control (Puerperal) shows the highest intensity level of the stain (level: 3). (B) Leukocyte alkaline phosphatase (LAP) scores in circulating granulocytes of peripheral blood from different categories of patients carrying JAK2V617F or CALR mutation. LAP values are shown in a scatter plot; diamond indicates the median. (i) LAP score levels of 38 PV, 74 ET, and 24 PMF patients carrying JAK2V617F mutant alleles. LAP values of all patients are above normal (>80). The Kruskal–Wallis test showed significant differences between the three disorders ($P < 0.001$ for all comparisons). (ii) LAP score levels of 18 ET patients and 1 PMF patient carrying CALR mutant alleles. The entire data showed no significant differences between the ET and PMF groups. Both ET and PMF patients carrying the CALR mutation exhibited lower LAP scores than the normal value. (iii) LAP score levels of 74 ET patients with JAK2V617F mutant alleles and 18 ET patients carrying the CALR mutation. The Mann–Whitney U test showed significant differences between both groups ($P < 0.001$). (iv) LAP score levels of 24 PMF patients with JAK2V617F mutant alleles and 1 PMF patient carrying the CALR mutation. These data show that LAP score levels of PMF patients with JAK2V617F mutation are markedly higher than those with the CALR mutation.

**Fig 1.** (A) LAP scoring images of CALR and JAK2V617F mutant patients. Peripheral blood smear of a representative CALR mutant patient displays a poor LAP expression (positive-cells:1%; score:1). In contrast, peripheral blood smear of a representative JAK2V617F mutant patient reveals a marked LAP expression (positive-cells:90%; score:194). Peripheral blood smear of a representative positive control (Puerperal) shows the highest intensity level of the stain (level: 3). (B) Leukocyte alkaline phosphatase (LAP) scores in circulating granulocytes of peripheral blood from different categories of patients carrying JAK2V617F or CALR mutation. LAP values are shown in a scatter plot; diamond indicates the median. (i) LAP score levels of 38 PV, 74 ET, and 24 PMF patients carrying JAK2V617F mutant alleles. LAP values of all patients are above normal (>80). The Kruskal–Wallis test showed significant differences between the three disorders ($P < 0.001$ for all comparisons). (ii) LAP score levels of 18 ET patients and 1 PMF patient carrying CALR mutant alleles. The entire data showed no significant differences between the ET and PMF groups. Both ET and PMF patients carrying the CALR mutation exhibited lower LAP scores than the normal value. (iii) LAP score levels of 74 ET patients with JAK2V617F mutant alleles and 18 ET patients carrying the CALR mutation. The Mann–Whitney U test showed significant differences between both groups ($P < 0.001$). (iv) LAP score levels of 24 PMF patients with JAK2V617F mutant alleles and 1 PMF patient carrying the CALR mutation. These data show that LAP score levels of PMF patients with JAK2V617F mutation are markedly higher than those with the CALR mutation.

**Fig 1.** (A) LAP scoring images of CALR and JAK2V617F mutant patients. Peripheral blood smear of a representative CALR mutant patient displays a poor LAP expression (positive-cells:1%; score:1). In contrast, peripheral blood smear of a representative JAK2V617F mutant patient reveals a marked LAP expression (positive-cells:90%; score:194). Peripheral blood smear of a representative positive control (Puerperal) shows the highest intensity level of the stain (level: 3). (B) Leukocyte alkaline phosphatase (LAP) scores in circulating granulocytes of peripheral blood from different categories of patients carrying JAK2V617F or CALR mutation. LAP values are shown in a scatter plot; diamond indicates the median. (i) LAP score levels of 38 PV, 74 ET, and 24 PMF patients carrying JAK2V617F mutant alleles. LAP values of all patients are above normal (>80). The Kruskal–Wallis test showed significant differences between the three disorders ($P < 0.001$ for all comparisons). (ii) LAP score levels of 18 ET patients and 1 PMF patient carrying CALR mutant alleles. The entire data showed no significant differences between the ET and PMF groups. Both ET and PMF patients carrying the CALR mutation exhibited lower LAP scores than the normal value. (iii) LAP score levels of 74 ET patients with JAK2V617F mutant alleles and 18 ET patients carrying the CALR mutation. The Mann–Whitney U test showed significant differences between both groups ($P < 0.001$). (iv) LAP score levels of 24 PMF patients with JAK2V617F mutant alleles and 1 PMF patient carrying the CALR mutation. These data show that LAP score levels of PMF patients with JAK2V617F mutation are markedly higher than those with the CALR mutation.

**Fig 1.** (A) LAP scoring images of CALR and JAK2V617F mutant patients. Peripheral blood smear of a representative CALR mutant patient displays a poor LAP expression (positive-cells:1%; score:1). In contrast, peripheral blood smear of a representative JAK2V617F mutant patient reveals a marked LAP expression (positive-cells:90%; score:194). Peripheral blood smear of a representative positive control (Puerperal) shows the highest intensity level of the stain (level: 3). (B) Leukocyte alkaline phosphatase (LAP) scores in circulating granulocytes of peripheral blood from different categories of patients carrying JAK2V617F or CALR mutation. LAP values are shown in a scatter plot; diamond indicates the median. (i) LAP score levels of 38 PV, 74 ET, and 24 PMF patients carrying JAK2V617F mutant alleles. LAP values of all patients are above normal (>80). The Kruskal–Wallis test showed significant differences between the three disorders ($P < 0.001$ for all comparisons). (ii) LAP score levels of 18 ET patients and 1 PMF patient carrying CALR mutant alleles. The entire data showed no significant differences between the ET and PMF groups. Both ET and PMF patients carrying the CALR mutation exhibited lower LAP scores than the normal value. (iii) LAP score levels of 74 ET patients with JAK2V617F mutant alleles and 18 ET patients carrying the CALR mutation. The Mann–Whitney U test showed significant differences between both groups ($P < 0.001$). (iv) LAP score levels of 24 PMF patients with JAK2V617F mutant alleles and 1 PMF patient carrying the CALR mutation. These data show that LAP score levels of PMF patients with JAK2V617F mutation are markedly higher than those with the CALR mutation.
higher than the normal LAP score. To compare the differences in LAP expression between CALR-mutated and JAK2V617F-mutated patients, we stratified patients into two different subgroups based on disease categories: ET group and PMF group. For the ET group, the median values of LAP scores in CALR-mutated and JAK2V617F-mutated patients were 17 and 142, respectively. Statistically significant difference was observed between the two mutation groups (P < 0.001) (Fig 1B). For the PMF group, data from only one PMF patient with CALR mutations were collected, which exhibited a lower LAP score of 11, and the median value in patients with JAK2V617F was 148. Although statistical analysis of these results was not performed because of the lack of sufficient samples, an apparent difference can be observed between scores from the two mutation groups (Fig 1B).

**Discussion**

Since the identification of CALR exon 9 mutations in patients with JAK2-unmutated MPN [7,8], different hematologic centers worldwide successively performed detection of CALR mutations in MPN patients. The differences between our findings and those from the other centers are listed in Table 1. The CALR mutation rate in our study was extremely lower than those in the other hematologic centers, regardless of whether the subjects come from Western countries or from the four different geographical sites in China. By contrast, the findings from the four hematologic centers in China showed close frequencies to those reported in Caucasian patients. This result suggests that ethnic differences are not apparent between Chinese and Caucasian patients with regard to the CALR mutation rate. From the currently available data, CALR mutation rates exhibited a geographical difference in China, although this difference may be affected by several factors. These factors include the diagnostic criteria and assays used. Another significant difference was noted in the percentages of triple-negative patients between the Chinese and Caucasian patients. The data obtained from the four centers in China, including those from our center but excluding those by Chen et al., showed that up to 20%–30% of Chinese patients with ET and 27%–48% of Chinese patients with PMF were triple-negative for the mutations. These frequencies are substantially higher than those reported in Caucasian patients with either ET or PMF. The large discrepancy may be attributed to the different frequencies of JAK2V617F, MPL, or CALR mutations, considering the unique genetic variation patterns of the three genes in different centers. Several of these differences may reflect the different sensitivities of the molecular approaches adopted, but the genetic and geographical differences by which ET and PMF develop may play more important roles. Genetic and geographical differences may imply that one or more undiscovered mutations are responsible for ET or PMF in a considerable proportion of patients with Chinese descent. Moreover, in recent years, a number of novel mutations including TET2, IDH1/2, Ezh2, DNMT3A and Asxl1 have been described in BCR-ABL1-negative MPNs. None of these mutations were MPN-specific. They are more frequent in post-PV/ET MF, PMF and blast-phase MPN, and coexist with JAK2, MPL and CALR mutations, indicating that these abnormalities could involved in disease clonal evolution or blastic transformation of MPNs [18–20]. It is not clear as to whether these mutations contribute to molecular pathogenesis of triple-negative Chinese patients with MPN.

Currently, more than 50 different CALR indels have been described. In this study, we also reported 10 types of novel CALR indel mutations. All reported CALR mutations, including the 10 types of novel mutations, shifted the reading frame by one base pair and consequently produced distinct C-terminal amino acid sequences. Therefore, further studies are necessary to explain the generation of the unique mutation patterns of the CALR gene and to define whether these diverse CALR mutations can lead to different potential biological and clinical effects in
Furthermore, in a study conducted in the Shanghai region of China [21], investigators found a number of scattered point mutations of the CALR gene in patients with and without the exon 9 indels. However, these somatic point mutations were not found in our center. Thus, we inferred that production of these point mutations may be closely associated with the environment in the Shanghai region.

Among all reported CALR indels, a 52 bp deletion (type 1 mutation) and a 5 bp insertion (type 2 mutation) were found in more than 80% of all CALR-mutant patients. Interestingly, the frequency of type 1 mutation was significantly higher in PMF than that in ET, suggesting a specific role of this mutation in myelofibrotic transformation [15,22]. This phenomenon is highly similar to that of the JAK2V617F mutation. Previous studies showed that JAK2V617F mutation is found in the majority of patients with PV and in many cases of ET or idiopathic myelofibrosis [1,2]. Several studies on biological function also confirmed that JAK2V617F mutation transfected into murine bone marrow cells produces erythrocytosis and subsequent myelofibrosis in recipient animals [1, 23,24]. This finding suggests that the JAK2V617F mutation prefers the expansion of the erythroid lineage. To further explore this issue, we compared our data with those of the other centers. As shown in Table 2, our study recorded a high frequency of type 1 CALR mutation and a low frequency of type 2 CALR mutation in PMF compared with ET. Data from two Western countries also showed the same results [7,8]. By contrast, studies conducted by Li et al. and Qiao et al. [11,13], reported that the frequency of type 2 mutation is significantly higher than that of type 1 mutation in PMF. Moreover, Chen et al. [14] reported that the frequency of type 1 mutation is significantly higher in ET. These conflicting results may be attributed to insufficient case numbers or unknown molecular mechanisms. Evidence necessary to explain such discrepancies is still lacking. The current data do not completely support previous results. Biological effects of type 1 and 2 mutations should be investigated by building murine models.

We compared the demographic and hematological characteristics of Chinese patients with ET or PMF according to their JAK2V617F and CALR genotypes (Table 3). ET patients carrying a CALR indel displayed unique hematological phenotypes that differed from those with JAK2V617F mutations, in particular, the high platelet counts, and low leukocyte counts and hemoglobin levels. These findings are almost identical to those of many other studies [7,8, 11–14,16,17] and indicate that CALR-mutated ET appears to be substantially different from JAK2-mutated ET in terms of biological and hematological features. In contrast to the other studies [7,8, 11–14,16,17], no predilection toward the male sex or a younger age in CALR-mutated ET patients was observed. This difference in demographic parameters is probably associated with the different case numbers. For PMF patients, significant differences were not found between Chinese patients with PMF with mutant CALR and those with JAK2V617F in terms of gender, age, leukocyte counts, platelet counts, or hemoglobin levels. By contrast, Rumi et al. [15] showed a younger age predilection, lower leukocyte counts, and higher platelet counts in a large number of CALR-mutated PMF patients (n = 140). The large discrepancy probably results from the small cohort sizes and the different ethnical backgrounds.

In addition, demographic and hematological characteristics of ET patients with different CALR type 1 and CALR type 2 mutations were analyzed (Table 4). Some differences were mainly observed in our data. Patients with CALR type 1 mutation or CALR type 2 mutation respectively share a characterization with lower leukocyte counts than those with JAK2V617F mutation. Moreover, female patients with CALR type 1 mutation and female patients with CALR type 2 mutation also both presented lower hemoglobin levels, compared with female patients with JAK2V617F mutation. These observations indicate that CALR mutation and JAK2V617F mutation exert different effect on leukocyte and erythroid cell. Patients with CALR

MPN.
type 2 mutation displayed higher platelet counts than those with JAK2V617F mutation. However, patients with CALR type 1 and CALR type 2 mutations displayed the similar leukocyte counts, hemoglobin levels, and platelet counts. Similar results were presented by Qiao et al. [13] but not evaluated by others. These findings indicate that patients with CALR type 2 mutation may present a phenotype associated with the preferential expansion of the megakaryocytic lineage. Overall, these results suggest that the disease biology varies considerably according to different genetic lesions.

LAP scoring is a useful tool for evaluating granulocyte activation. LAP, which was classically named as neutrophil alkaline phosphatase, is stored in secretory vesicles of circulating neutrophils. Acquisition of LAP enzymatic activity is a late event during myeloid maturation and indicates that the enzyme is expressed in mature neutrophils. Hence, LAP is an indispensable marker of functionally mature neutrophils [25–27]. Passamonti et al. [28] found that neutrophils carrying the JAK2V617F mutation expressed higher levels of LAP in all types of MPN than healthy controls, thus suggesting that JAK2V617F may constitutively activate granulocytes. In the present study, we compared LAP expression characteristics of the MPN patients who were categorized according to their JAK2V617F and CALR genotypes. Fig 1 shows that LAP scores were markedly elevated in all types of MPN patients with JAK2V617F mutation, but frequently decreased in MPN patients with CALR mutation. As mentioned earlier, the evident differences observed among the diverse genetic subtypes suggest that the granulocyte biology in MPN patients varies considerably according to different genetic lesions. Recent research [7,8] confirmed that JAK–STAT (Janus kinase–signal transducer and activator of transcription) signaling is involved in the cytokine-independent growth of mutant CALR-expressing Ba/F3 cells. Seido oku et al. [26] confirmed that JAK2V617F signaling stimulates LAP expression in neutrophils, specifically by activating STAT3-dependent signaling pathway in vitro. JAK2V617F signaling promotes cell proliferation through STAT5 activation [26]. However, findings of this study also indicate that mutant calreticulin likely results in unknown abnormalities in the metabolic pathways of neutrophils.

In JAK2V617F-positive MPN patients, we found that LAP expression was also distinct among disease categories. Highest LAP levels were found in patients with PV, followed by patients with PMF and then with ET. A similar observation was previously noted by Basquiera et al. [29]. Such finding is consistent with previous studies [26,28] wherein patients carrying homozygous mutations exhibited higher levels of neutrophil LAP than those carrying heterozygous mutations, suggesting that LAP levels in neutrophils depend on the levels of JAK2V617F expression. Homozygous mutation for JAK2V617F is more frequent in PV than in ET or PMF. Thus, the highest LAP score in patients with PV may be due to a greater prevalence of homozygosity for JAK2V617F mutation in PV. Overall, our results strengthen the findings previously reported by other groups.

In conclusion, we reported a relatively lower mutation rate of CALR gene and a higher triple-negative frequency in Chinese MPN patients than in Caucasian patients. These data imply that a genetic alteration may be involved in up to 48% of triple-negative Chinese patients with ET or PMF. Hence, investigation of mutant molecules in our mutation-negative patients should be further investigated using exomic and whole genome sequencing. We also revealed that Chinese ET patients with CALR indels exhibited unique phenotypes similar to Caucasian ET patients, including low Hb levels, low WBC counts, and high platelet counts. Our data showed that patients with CALR type 2 mutation may present a phenotype associated with the preferential expansion of the megakaryocytic lineage, which is in contrast to those with CALR type 1 mutation. This study is the first to report that patients carrying CALR indels have relatively low LAP expression, which is a unique phenotype that is discrepant from those with JAK2V617F.
Acknowledgments

We would like to thank Jian Sa (Shanxi Medical University) who conducted the statistical analyses in this study.

Author Contributions

Conceived and designed the experiments: HWW HXG XHC. Performed the experiments: HXG XHC RYT JMC JLL. Analyzed the data: HXG XHC HWW. Contributed reagents/materials/analysis tools: YHT ZFX FGR JXZ JP NZ XJW JXH WFY. Wrote the paper: HXG XHC.

Recruited the patients: YHT ZFX FGR JXZ JP NZ XJW JXH WFY.

References

1. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005; 365:1054–1061. PMID: 15781101
2. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signaling causes polycythemia vera. Nature. 2005; 434:1144–1148. PMID: 15793561
3. Pardanani AD, Lasho TL, Finke C, Hanso CA, Tefferi A. Prevalence and clinicopathologic correlates of JAK2 exon12 mutations in JAK2V617F negative polycythemia vera. Leukemia. 2007; 21:3472–3476. PMID: 17597810
4. Scott LM, Wong W, Levine RL, Scott MA, Beer PA, Stratton MR, et al. JAK2 exon 12 mutations in poly- cytopenia vera and idiopathic erythrocytosis. N Engl J Med. 2007; 356:459–468. PMID: 17267906
5. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadeleigh M, et al. MPLW515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006; 108:3472–3476. PMID: 16968251
6. 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. PMID: 16834459
7. 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; 369:2379–2390. doi:10.1056/NEJMo1311347 PMID: 24323556
8. Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, et al. Somatic CALR mutations in myeloproliferative epotheses with nonmutated JAK2. N Engl J Med. 2013; 369:2391–2405. doi: 10.1056/NEJMo1311294 PMID: 24323539
9. Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myelo- proliferative neoplasms: order out of chaos. Cancer. 2009; 115:3842–3847. doi: 10.1002/cncr.24440 PMID: 19472396
10. Chen X, Qi X, Tan Y, Xu Z, Xu A, Zhang L, et al. Detection of MPL exon10 mutations in 103 Chinese patients with JAK2V617F-negative myeloproliferative neoplasms. Blood Cells Mol Dis. 2011; 47:67–71. doi: 10.1016/j.bcmd.2011.04.004 PMID: 21555228
11. Li B, Xu J, Wang J, Gale RP, Xu Z, Cui W, et al. Calreticulin mutations in Chinese with primary myelofi- brosis. Haematologica. 2014; 99:1697–1700. doi: 10.3324/haematol.2014.109249 PMID: 24997152
12. Shen H, Chao H, Ding Z, Feng Y, Cen J, Pan J, et al. CALR and ASXL1 mutation analysis in 190 patients with essential thrombocytemia. Leuk Lymphoma. 2015; 56:820–822. doi: 10.3109/10428194.2014.939963 PMID: 25005031
13. Qiao C, Sun C, Ouyang Y, Wang JJ, Qian SX, Li JY, et al. Clinical importance of different calreticulin gene mutation types in wild-type JAK2 essential thrombocytemia and myelofibrosis patients. Haematologica. 2014; 99:e182–184. doi: 10.3324/haematol.2014.109199 PMID: 25015940
14. Chen CC, Gau JP, Chou HJ, You JY, Huang CE, Chen YY, et al. Frequencies, clinical characteristics, and outcome of somatic CALR mutations in JAK2-unmutated essential thrombocytemia. Ann Hematol. 2014; 93:2029–2036. PMID: 25015052
15. Rumi E, Pietra D, Pascutto C, Guglielmelli P, Martinez-Trillos A, Casetti I, et al. Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis. Blood. 2014; 124:1062–1069. doi: 10.1182/blood-2014-05-578435 PMID: 24986690
16. 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; 123:1544–1551. doi:10.1182/blood-2013-11-539098 PMID: 24366362

17. Andrikovics H, Krahlting T, Balassa K, Halm G, Bors A, Koszarska M, et al. Distinct clinical characteristics of myeloproliferative neoplasms with calreticulin mutations. Haematologica. 2014; 99:1184–1190. doi: 10.3324/haematol.2014.107482 PMID: 24895336

18. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia, 2010 Apr 29.

19. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carubbia N, et al. Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. Genes Chromosomes Cancer. 2012 Aug; 51(8):743–55. doi:10.1002/gcc.21960 PMID: 22489043

20. Chen E, Schneider RK, Breyfogle LJ, Rosen EA, Poveromo L, Elf S, et al. Distinct effects of concomitant Jak2V617F expression and Tet2 loss in mice promote disease progression in myeloproliferative neoplasms. Blood. 2015 Jan 8; 125 (2): 327–35. doi: 10.1182/blood-2014-04-567024 PMID: 25281607

21. Wu Z, Zhang X, Xu X, Chen Y, Hu T, Kang Z, et al. The Mutation Profile of JAK2 and CALR in Chinese Han Patients with Philadelphia Chromosome-Negative Myeloproliferative Neoplasms. Journal of Hematology & Oncology. 2014; 7: 48.

22. Cabagnols X, Defour JP, Ugo V, Ianotto JC, Mossuz P, Mondet J, et al. Differential association of calreticulin type 1 and type 2 mutations with myelofibrosis and essential thrombocytemia: relevance for disease evolution. Leukemia. 2015; 29: 249–252. doi: 10.1038/leu.2014.270 PMID: 25212275

23. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood. 2006; 108:1652–1660. PMID: 16670266

24. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of JAK2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006; 107: 4274–4281. PMID: 16774797

25. Garattini E, Gianni M. Leukocyte alkaline phosphatase a specific marker for the post-mitotic neutrophilic granulocyte: regulation in acute promyelocytic leukemia. Leukaemia & Lymphoma. 1996; 23: 493–503.

26. Oku S, Takenaka K, Kuriyama T, Shide K, Kumano T, Kikushige Y, et al. JAK2V617F uses distinct signaling pathways to induce cell proliferation and neutrophil activation. British Journal of Haematology. 2010; 150: 334–344. doi: 10.1111/j.1365-2141.2010.08249.x PMID: 20553273

27. Stewart CA. Leucocyte alkaline phosphatase in myeloid maturation. Pathology. 1974; 6:287–293. PMID: 4528632

28. Passamonti F, Rumi E, Pietra D, Della Porta MG, Boveri E, Pascutto C, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood. 2006; 107: 3676–3682. PMID: 16373657

29. Basquiera AL, Fassetta F, Soria N, Barral JM, Ricchi B, García JJ. Accuracy of leukocyte alkaline phosphatase score to predict JAK2V617F mutation. Haematologica. 2007; 92: 704–705. PMID: 17488700