LETTER TO THE EDITOR

Frequent CALR exon 9 alterations in JAK2 V617F-mutated essential thrombocythemia detected by high-resolution melting analysis

Essential thrombocythemia (ET) is a clonal hematopoietic stem cell neoplasm and one of the classic BCL-ABL1-negative chronic myeloproliferative neoplasm (MPN), which also includes polycythemia vera and primary myelofibrosis (PMF). Recently, two seminal studies discovered a high frequency of somatic calreticulin (CALR) mutations in patients with JAK2/MPL-unmutated ET and PMF. The pattern of most CALR mutations in MPN is heterozygous indels in exon 9 causing one-base pair (bp) reading frameshift. CALR mutations have been shown to have important diagnostic and prognostic significance in ET and PMF patients, and will likely be incorporated into the World Health Organization (WHO) diagnostic criteria for MPN. In vitro studies on the molecular pathogenesis of CALR mutations in MPN have shown controversial results in regard to the involvement and/or activation of the JAK/STAT signaling pathway, and the exact pathogenesis of CALR mutations is not yet completely understood at the present time.

Several techniques such as Sanger sequencing and polymerase chain reaction (PCR) followed by fragment analysis have been used to detect CALR mutations. High-resolution melting analysis (HRMA) is a well-established method for the screening of mutations, and we have developed a rapid and sensitive HRMA for the detection of CALR exon 9 mutations. In this study, we sought to screen a cohort of 92 Taiwanese ET patients for CALR exon 9 mutations with HRMA and Sanger sequencing independently, and to determine the clinical and molecular correlates.

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After screening the 59 JAK2 V617F-mutated ET patients for CALR alterations by HRMA, 16 (27.1%) samples were found to have distinct melting curves from wild type (Figure 1). In 2 of these 16 samples, one CALR type 3 mutation (p.L367fs*48) and one single-nucleotide polymorphism (rs143880510) were detected using Sanger sequencing. All the other 14 samples were wild type by sequencing. Interestingly, we detected a high frequency of CALR exon 9 alterations in 12 (85.7%) of these 14 patients after TA-cloning (Table 1A). Three patients harbored the classic CALR indel mutations: one each of type 2 p.K385fs*47, p.E370fs*60 and p.E371fs*59. Hence, four (6.8%) ET patients had classic CALR indel and JAK2 V617F co-mutations in this cohort. Five patients (8.5%) including the aforementioned patient (P520) with type 2 CALR mutation harbored four types of 3-bp inframe deletions all resulted in the deletion of a single amino acid of glutamic acid: two p.E381del and one each of p.E371del, p.E378del and p.E396del (Supplementary Figure 1). Another five patients (8.5%) harbored five types of point mutations: one each of p.E374X, p.E380X, p.K391X, p.E372G and p.E380G. The latter p.E380G has been reported as a single-nucleotide polymorphism but might be a low-allele-burden somatic mutation in this patient because it was only detected after TA-cloning and not by Sanger sequencing on patient’s genomic DNA. The remaining two patients were found to have wild-type CALR exon 9 after screening for 100 independent clones, and were counted as CALR wild type. Overall, various CALR exon 9 alterations were detected in 13 (22%) of 59 JAK2 V617F-mutated ET patients.

We then examined the clinical and molecular correlates in 91 ET patients excluding the one MPL-mutated patient (Table 1B). JAK2-mutated ET patients with concomitant CALR alterations were associated with oldest age (P=0.025), higher thrombotic events after diagnosis (P=0.048), higher major arterial thrombotic events after diagnosis (P=0.022) and more patients being in the high-risk group for thrombohemorrhagic complications (P=0.03). Consistent with previous reports, CALR mutations were associated with younger age (P=0.025), higher platelet count (P<0.0001) and lower hemoglobin level (P=0.016). JAK2 V617F mutation was associated with leukocytosis (P=0.046).

After the discovery of CALR mutations, it has been proposed to be mutually exclusive with JAK2 and MPL mutations in MPN. However, CALR and JAK2 V617F co-mutations have been reported in a few MPN cases across different ethnic groups and the frequency is usually below 1%. In contrast to these reports, we detected a higher frequency of 6.8% CALR indel and JAK2 co-mutations in ET patients. Interestingly, three of these CALR mutations were low-allele-burden mutants not detected using Sanger sequencing. Nevertheless, the use of a sensitive HRMA technique has enabled us to detect these low-allele-
Table 1B. Variables All (n = 91) A. JAK2 V617F mutation (n = 46) B. CALR mutation (n = 21) C. JAK2-mutated and CALR alterations (n = 13) D. Triple-negative (n = 11) A vs B vs C vs D, F test, p value

| Variables | All (n = 81) | A vs B | A vs C | B vs C | A vs D | B vs D | C vs D |
|-----------|-------------|--------|--------|--------|--------|--------|--------|
| Male/female gender, (%) | 39/52 (43/57) | 21/25 (46/54) | 9/12 (43/57) | 5/8 (39/61) | 4/7 (36/64) | NS | NS | NS | NS |
| Age at diagnosis (years), median (range) | 53 (22–89) | 54.5 (25–89) | 47 (22–76) | 60 (26–80) | 52 (35–79) | 0.025 | 0.012 | NS | 0.004 |
| Follow-up (years), median (range) | 3.7 (0.02–23.1) | 5.4 (0.05–23.1) | 3.8 (0.02–6.1) | 2.7 (0.02–10.4) | 3.1 (0.02–10.3) | 0.025 | 0.022 | NS | 0.009 |
| History of thrombosis, (%) | 19 (20.9) | 9 (19.6) | 3 (14.3) | 5 (38.5) | 2 (18.2) | NS | NS | NS | NS |
| Major thrombosis, (%) | 17 (18.7) | 8 (17.4) | 2 (9.5) | 5 (38.5) | 2 (18.2) | NS | NS | NS | NS |
| Thrombosis after diagnosis, (%) | 6 (6.6) | 1 (2.2) | 1 (4.8) | 3 (23.1) | 1 (9.1) | NS | 0.022 | 0.03 | NS |
| Major arterial thrombosis after diagnosis, (%) | 9 (p.E393_E395del and p.E405del) | 5 (p.E393_E395del and p.E405del) | 2 (p.E393_E395del and p.E405del) | 2 (p.E393_E395del and p.E405del) | 1 (p.E393_E395del and p.E405del) | NS | NS | NS | NS |
| History of hemorrhage, (%) | 25 (27.5) | 13 (28.6) | 9 (42.9) | 2 (15.4) | 1 (9.1) | 0.016 | 0.016 | NS | NS |
| Major hemorrhage, (%) | 17 (18.7) | 9 (19.6) | 6 (28.6) | 2 (15.4) | 0 NS | NS | NS | NS | NS |
| Hemoglobin (g dl−1), median (range) | 9.2 (4.9–24.2) | 11.8 (6.0–24.2) | 8.2 (5.3–24.2) | 12.8 (9.3–15.2) | 11.8 (6.0–24.2) | NS | NS | NS | NS |
| WBC (×10^3 μl−1), median (range) | 936 (335–2834) | 942 (335–1496) | 1351 (642–2834) | 855 (547–1931) | 708 (532–1496) | 0.001 | 0.023 | NS | 0.001 |
| Platelets (×10^9 l−1), median (range) | 302 (90–999) | 301 (90–999) | 243 (60–429) | 832 (263–1789) | 472 (151–1179) | NS | NS | NS | NS |

Abbreviations: n, number; NS, not significant; WBC, white blood cell. aHigh-risk group for thrombohemorrhagic complications: Age ≥ 60 years and/or a previous history of thrombosis.

In conclusion, we have detected a high frequency of both classic and non-classic CALR exon 9 alterations in JAK2-mutated ET patients by HRMA. The presence of CALR alterations in JAK2-mutated ET defines a specific subgroup of patients requiring careful follow-up and management for their increased risk of thrombotic events. Because our study is limited by small patient number, larger study is warranted to confirm our observation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

K-HL, CG-SC, Y-YK and W-CC conceived of the study, participated in its design and/or coordination, and edited the manuscript. K-HL, H-CL, CG-SC, Y-YK, H-IC, N-WS, JL, Y-FC, M-CC and R-KH enrolled patients into the study. K-HL and W-TW carried out experiments and data analysis. K-HL and W-TW drafted the manuscript. All authors approved the manuscript.

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