

LETTER TO THE EDITOR

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

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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.^{2,3} 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, 2and 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, 2,3 and the exact pathogenesis of CALR mutations is not yet completely understood at the present time.5

Several techniques such as Sanger sequencing and polymerase chain reaction (PCR) followed by fragment analysis have been used to detect *CALR* mutations.^{2,3,6,7} 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.⁸ 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.

The institutional review board of Mackay Memorial Hospital has approved the screening for mutations. All patients provided written informed consent. Diagnosis of ET was established on the basis of the 2008 WHO criteria. The clinical and laboratory characteristics at the time of diagnosis or referral were collected. Genomic DNAs derived from the bone marrow, peripheral blood and peripheral blood granulocytes and/or mononuclear cells were used for mutation screening. CALR mutations were screened by Sanger sequencing on an ABI 3730 sequencer as preciously described.3 CALR exon 9 mutations were independently screened by HRMA using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described with a maximal sensitivity of 2.5% for both CALR type 1 and type 2 mutants.⁸ Briefly, a pair of oligonucleotide primers were used to amplify a 134-bp amplicon (GenBank: NM_004343), which flanked all CALR exon 9 variants reported in MPN. All samples with distinguished melting curves from wild type were confirmed by duplicate studies. Peripheral blood samples from 78 healthy adults were also used to validate the specificity of our HRMA. JAK2 V617F mutation was screened using allele-specific PCR with an analytic sensitivity of 5% and MPL exon 10 mutation using Sanger sequencing as previously described. 9,10 TA-cloning was performed using the pGEM-T easy vector system (Promega, Madison, CA, USA) as previously described.8 At least 10 clones in each individual were randomly selected for the screening of CALR exon 9 alterations by Sanger sequencing. All novel single-nucleotide variant that was only detected once was treated as artifact and were excluded. The SPSS Statistics software (IBM, New York, NY, USA) was used for all calculations. P-values < 0.05 were considered significant.

Among the 92 ET patients (median age 53 years; 58% females), 59 (64%) patients harbored *JAK2* V617F mutation and one (1%)

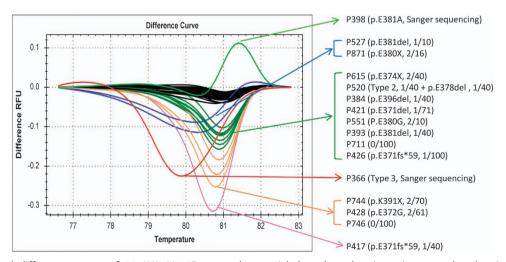


Figure 1. Normalized difference curves of 16 *JAK2* V617F-mutated essential thrombocythemia patient samples showing distinct melting curves from *CALR* exon 9 wild-type samples (black color). Corresponding patient number, genotype and number of positive clone in TA-cloning of each curve is indicated by arrow.



patient harbored MPL W515K mutation. Thirty-two JAK2/MPLunmutated ET patients were utilized for the development of our HRMA platform. 8 Briefly, 22 (68.8%) samples were found to have distinct melting curves from wild type. In 16 of these 22 samples, Sanger sequencing confirmed the presence of six types of CALR mutations: five type 1 (p.L367fs*46), six type 2 (p.K385fs*47), one type 3 (p.L367fs*48), two type 34 (p.K385fs*47) and two other types (p.L367fs*43 and p.E369fs*50). The other six samples were wild type by sequencing, and CALR type 2 mutations were detected in five of six patients after TA-cloning, indicating the presence of low-allele-burden CALR mutants in them. By using our HRMA platform, we identified CALR mutations in 21 (22.8% overall and 65.6% in JAK2/MPL-unmutated) ET patients and this frequency is comparable to other studies.²⁻⁴ Eleven (12%) ET patients were negative for JAK2, CALR and MPL mutations. In the 78 samples from healthy adults, two were found with HRMA to have distinct melting curves from wild type. One single-nucleotide polymorphism (rs143880510) and one wild type were found after Sanger sequencing in these two samples. Therefore, our HRMA system has a low false-positive rate of 1.3%.

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 singlenucleotide 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 TAcloning (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 an 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=0.023). Consistent with previous reports, *CALR* mutations were associated with younger age (P=0.025), higher platelet count (P<0.001) 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%.^{7,11–13} 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 1A	. CALR exon	9 alterations and single	-nucleotide p	olymorp	Table 1A. CALR exon 9 alterations and single-nucleotide polymorphism in 14 JAK2 V617F-mutated essential thrombocythemia patients detected using high-resolution melting analysis	d using high-r	esolution meltin	y analysis
Patient	CALR mutation	Nucleotide change	Protein change	Amino acid	Protein sequence ^a	CALR SEQ	CALR-TA clone number ^b	JAK2 V617F allele burden ^c
N	Wild type	AN	NA	417	QRLKEEBEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	ΥZ	NA
P520	Type 2	c.1154_1155insTTGTC	p.K385fs ^a 47	430	QRLKEEEEEDKKRKEEEEEAEDNCRRMMRTKMRMRRRRRRRRKMRRKMSPARPRTSCREACLQGWTEA	Wild type	1/40	2%
P366	Type 3	c.1095_1140del (∆46)	p.L367fs ^a 48	413	QRQRTR <u>RMMRTKMRRRRRRRRRRRRRRRRRRRRRRRRRRRRR</u>	Heterozygous	NA	83%
P426	New	c.1108delG (Δ1)	p.E370fs ^a 60	428	QRLKERKKTRNAKRRRRQRTR <u>RMMRTKMRMRRRTRRKMRRKMSPARPRTSCREACLQGWTEA</u>	Wild type	1/100	25%
P417	New	c.1111delG (Δ1)	p.E371fs ^a 59	428	QRLKEEKKTRNAKRRRRQRTR <u>RMMRTKMRMRRRRRRRKMRRKMSPARPRTSCREACLQGWTEA</u>	Wild type	1/40	20%
P421	New	c.1110_1112delGGA (∆3)	p.E371del	416	QRLKEE-EDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/71	71%
P520	New	c.1132_1134delGAG (∆3)	p.E378del	416	QRLKEEEEEDKKRK-EEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	2%
P393	New	c.1142_1144delAGG (∆3)	p.E381del	416	QRLKEEEEEDKKRKEEE-AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	2%
P527	New	c.1142_1144delAGG (∆3)	p.E381del	416	QRLKEEEEDKKRKEEE-AEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	1/10	4%
P384	New	c.1188_1190delGGA (∆3)	p.E396del	416	QRLKEEEEEDKKRKEEEEEAEDKEDDEDKDEDE-DEEDKEEDEEEDVPGQAKDEL	Wild type	1/40	27%
P615	New	c.1120A>T	p.E374X	373	QRLKEEEED	Wild type	2/40	23%
P871	New	c.1138 G>T	p.E380X	379	QRLKEEEEEDKKRKEE	Wild type	2/16	32%
P744	New	c.1171A>T	p.K391X	390	QRLKEEEEEDKKRKEEEEEAEDKEDDED	Wild type	2/70	13%
P428	New	c.1115A > G	p.E372G	417	QRLKEEEGDKKRRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	2/61	20%
P551	rs201971744	c.1139A > G	p.E380G	417	QRLKEEEEEDKKRKEEGEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Wild type	2/10	41%
P398	rs143880510	c.1142A > C	p.E381A	417	QRLKEEEEDKKRKKEEEAAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	Heterozygous	NA	76%

sequence changes after +1 base pair reading frameshift. ^TA clones of the CALR PCR products amplified from each colony were analyzed using Sanger sequencing. The total number of clones examined and the number of each genotype are listed in the table. ^CBased on the relative peak areas of the mutant and wild-type PCR products in Sanger sequencing. All patients were tested positive for JAK2 V617F mutation ^aRed and blue fonts indicate acidic and basic amino acids, respectively. Underline indicates the same C-terminal sequencing. All patients Abbreviations: NA, not available; PCR, polymerase chain reaction; SEQ, Sanger sequencing. number of each genotype are listed in the table. ' using allele-specific PCR.

Table 1B.	Table 18. Clinical and laboratory characteristics at diagnosis or referral of 91 essential thrombocythemia patients stratified by mutation profiles	at diagnosis or ref	erral of 91 essential throm	bocythemia patients	stratified by mutation	ı profiles				
Variables		All (n = 91)	A. JAK2 V617F mutation B. CALR mutation $(n = 46)$ $(n = 21)$	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 P value	A vs B vs C P value	A vs B A vs C B vs C vs C P value P value o value	B vs C value
Male/fen	Male/female gender, n (%)	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 d	Age at diagnosis (years), median (range)	53 (22–89)	54.5 (25–89)	47 (22–76)	(26–80)	52 (35–79)	0.025	0.012	NS	0.004
Follow-u	Follow-up (years), median (range)	3.7 (0.02–23.1)	3.6 (0.04–23.1)	5,4 (0.5–13.2)	3.8 (0.02–6.1)	3.1 (0.2–10.3)	NS	NS	NS	0.032
History c	History of thrombosis, n (%)	19 (20.9)	9 (19.6)	3 (14.3)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Major th	Major thrombosis, <i>n</i> (%)	17 (18.7)	8 (17.4)	2 (9.5)	5 (38.5)	2 (18.2)	NS	NS	NS	NS
Thrombo	Thrombosis after diagnosis, n (%)	10 (11)	3 (6.5)	2 (9.5)	4 (30.8)	1 (9.1)	NS	0.048	0.036	NS
Major ar	Major arterial thrombosis after diagnosis, n (%)	(9.9)	1 (2.2)	1 (4.8)	3 (23.1)	1 (9.1)	NS	0.022	0.03	NS
History C	History of hemorrhage, n (%)	25 (27.5)	13 (28.3)	9 (42.9)	2 (15.4)	1 (9.1)	NS	NS	NS	NS
Major he	Major hemorrhage, <i>n</i> (%)	17 (18.7)	9 (19.6)	6 (28.6)	2 (15.4)	0	NS	NS	NS	NS
High-risk	High-risk group for thrombohemorrhagic	43 (47.3)	22 (47.8)	6 (28.6)	10 (76.9)	5 (45.5)	NS	0.023	NS	0.012
complica	complications", n (%)									
Hemoglc	Hemoglobin (g dl $^{-1}$), median (range)	13.3 (4.5–17.9)	14.0 (4.5–17.9)	12.6 (8.5–15.2)	13.3 (8.8–16.6)	12.8 (9.3–15.2)	0.016	0.016	NS	NS
WBC (x1	WBC $(x10^3 \mu l^{-1})$, median (range)	10.3 (4.8–29.9)	12.1 (4.8–29.9)	9.2 (4.9–27.9)	11.8 (6.0–24.2)	8.2 (5.3–25.5)	0.046	NS	NS	NS
Platelets	Platelets $(x_1^0)^{-1}$, median (range)	936 (335–2834)	942 (335–1496)	1351 (642–2834)	855 (547–1931)	708 (532–1374)	< 0.001	< 0.001	NS	0.001
Abbreviati	Abbreviations: n, number; NS, not significant; WBC, white blood cell. ^a High-risk group for thrombohemorrhagic complications: Age >60 years and/or a previous history of thrombosis	white blood cell. ^a F	High-risk group for thrombo	hemorrhagic complica	ations: Age ≥60 years a	nd/or a previous histo	ory of throm	bosis.		

burden CALR mutants in both JAK2-mutated and JAK2/MPLunmutated ET patients. In addition, we also detected several CALR exon 9 point mutations and inframe deletions in JAK2-mutated ET patients, but none in our JAK2/MPL-unmutated ET patients. Recently, point mutations in CALR were also reported in follicular lymphoma (E403X and E405Q), PMF (E379D) and chronic neutrophilic leukemia (E398D). 14 Two rare inframe deletions in *CALR* exon 9 (p.E393 E395del and p.E405del) have been reported in the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project with undetermined significance. All the five inframe deletions we detected were 3-bp deletions similar to the latter one. Although the possibility of low-allele-burden germline sequence variations cannot be completely excluded, these 3-bp inframe deletions detected using HRMA were more likely to be lowallele-burden somatic mutations not detected using Sanger sequencing in our patients. Recently, CALR point mutations (E381A and D373M) and inframe deletions (E381_A382 > A, D397_D400 > D, D400_K401 > D and E405_V409 > V) were also detected in patients with suspected MPN and JAK2-mutated MPN in another study albeit with a lower frequency.¹⁵ These CALR alterations were also found to co-occur with MPL, CSF3R, ASXL1 and ZRSR2. Currently, the role of these CALR point mutations and inframe deletions in the molecular pathogenesis of MPN is not yet clear. Because they frequently co-occurred with mutations involving the JAK-STAT pathway and affected disease phenotype in JAK2mutated ET patients, these non-classic CALR mutant proteins are suspected to have a contributory role in the pathogenesis of MPN.¹⁵ The frequency of these non-classic CALR mutations in PMF and other MPN requires further study.

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-CC, Y-HC, 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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