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Scientific Letter

Calreticulin Mutation Survey by High Resolution Melting Method Associated with Unique Presentations in Essential Thrombocythemic Patients

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To the editor.

Myeloproliferative neoplasms (MPN), characterized by abnormal proliferation of myeloid series, were found to have specific molecular markers.[1-5] In Philadelphia-negative and BCR-ABL-negative patients, JAK2 V617F mutation is the major marker and is present in approximately 90% in polycythemia vera patients and about 50~60% of patients with essential thrombocythemia (ET) and primary myelofibrosis (MF) patients.[1-4] The identification of this mutation contributes to the diagnosis and prognostic significance, as reported in many articles.[1-4] More recently, several investigations identified novel somatic mutations at the exon 9 of calreticulin (CALR) gene in 50~80% of JAK2V617F-negative ET and MF patients.[5]

CALR is a highly conserved endoplasmic reticulum calcium-binding chaperone that is related to calcium homeostasis, cell adhesion, and immune response.[9] Until now, all CALR mutations have been found at exon 9, which encodes the C-domain region where there is a domain for Ca2+ to bind.[5-9,14,15] Of the more than 50 mutations found, all were exclusively on JAK2 V617F-negative patients. Most of the mutations correspond to a 52 kb deletion (del 1092-1143, type 1) or a 5bp insertion (1151 ins TTGTC, type 2), resulting in premature terminations from these frameshift mutations.[7,8] From previous studies, the CALR mutations had a unique clinical presentation compared with JAK2 V617F mutation; therefore, it is essential to survey these mutations in Philadelphia chromosome and/or BCR-ABL negative MPNs.[10-14]

High resolution melting (HRM) method using the saturating dsDNA binding dye for melting curve analysis is a rapid and labor-saving method for mutation screening.[15] In this study, we used the HRM method to screen for these mutations in our ET patients by distinguishing the specific curve types. We also reviewed the clinical presentations of patients to find the relationship between mutations and clinical phenotypes.

Materials and methods.

Patients and samples. A total of 60 consecutive patients diagnosed with ET, according to Polycythemia vera Study Group criteria at Kaohsiung Medical University Chung-Ho Memorial Hospital, were enrolled in the study with informed consent. The DNA from whole peripheral blood was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The quality and concentration of DNA were determined by NanoVue Plus Spectrophotometer (GE Healthcare, UK) and stored at -20 °C for further use.

JAK2 V617F mutation survey. The JAK2 V617F mutation was identified by amplification refractory mutation system (ARMS).[4]

CALR mutation survey. For the HRM survey, the reaction was performed by the ABI ViiA7 machine. Briefly, 20 ng of DNA and 5 μM primers were amplified with the final volume of 20 μl with HRM reaction kit (MeltDoctor HRM Master Mix, Thermo fisher scientific, Waltham, MA, USA). The primer sets were designed (Primer Express® Software 3.0, Thermo fisher scientific, Waltham, MA, USA) with forward: 5'-GGCCTCTCTACAGCTCGTCCTT-3' and reverse 5'-ACGTCCGTCGTCTCTTTGTT-3'. The amplification conditions are as follows: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 62 °C for 1 min, with the acquisition of fluorescent signals at the end of each extension step. The fluorescent signals for HRM analysis were detected at 0.2 °C intervals, with hold-

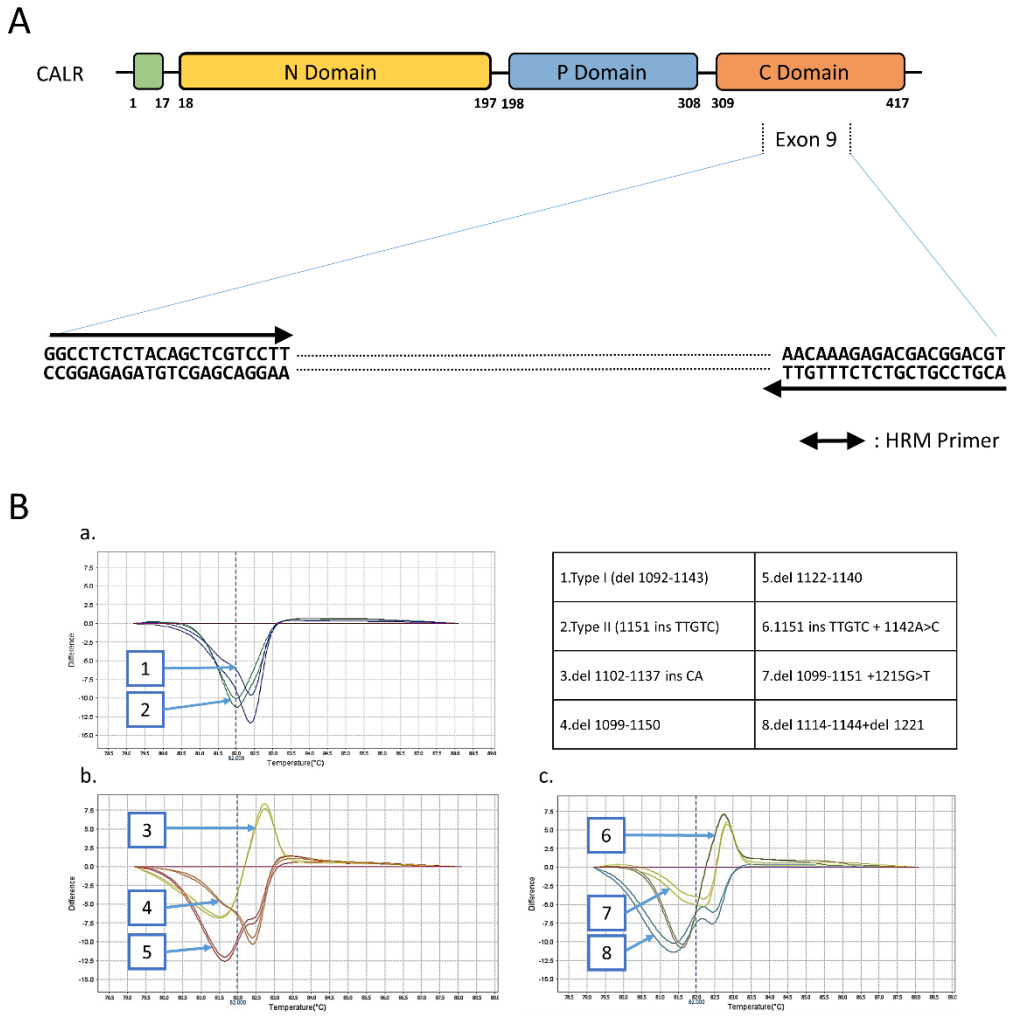
time for 10 s, between 95-60 °C. The selected primer set amplified 213 bp amplicon, and results were analyzed as fluorescence versus temperature graphs by software with normalized, temperature-shifting melting cures display as a difference plot.

For sequencing analysis, the DNA samples were carried out polymerase chain reaction (PCR) used with primer sets of forward: 5'-ACAACTTCCTCATCACCAACG-3' and reverse: 5'-GGCCTCAGTCCAGCCCTG-3'. The amplicons were performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) by bidirectional sequencing and analyzed by ABI 3730 XL DNA sequencing analyzer (Applied Biosystems, CA, USA). The sequencing results were compared to reference sequences of the CALR gene available on GenBank (www.ncbi.nlm.nih.gov/genbank/) to verify the difference.

Statistical Analysis. Statistical analyses were performed by SPSS software ver.19 for Windows (SPSS Inc., Chicago, IL, USA). The chi-square test was used to test categorical variables, while the Student's t-test compared continuous variables. A *p*-value of < 0.05 considered statistically significant.

Results. Of the ET patients studied, 96.6% have somatic mutations with JAK2 V617F and CALR mutations. Specifically, of the 60 ET patients studied, 34 (56.7%) were JAK2 V617F positive. Of the 26 JAK2V617F negative patients, 21 (80.8%) had CALR mutations were detected by HRM analysis with eight types of melting curves (**Figure 1**). These CALR mutations, confirmed by direct sequencing method, spread over exon 9, including del 1092-1143 mutations (type 1, 6 persons; 28.5%), 1151 ins TTGTC mutations (type 2, 7 patients; 33.3%), 3 complicated mutations and 3 other mutations (**Table 1**).

The clinical presentation revealed that there was no



1.Type I (del 1092-1143)	5.del 1122-1140
2.Type II (1151 ins TTGTC)	6.1151 ins TTGTC + 1142A>C
3.del 1102-1137 ins CA	7.del 1099-1151 +1215G>T
4.del 1099-1150	8.del 1114-1144+del 1221

Figure 1A. Schematic view of CALR gene and the HRM primer sets at exon 9. **Figure 1B** The curve patterns of CALR exon 9 by the HRM method. a. The melting peaks of 1 and 2 showed CALR exon 9 del 1092-1143 (type 1) and 1151 ins TTGTC (type 2). b. CALR exon 9 del 1102-1137 ins CA, del 1099-1150, and del 1122-1140 were showed in HRM melting cures of 3, 4, and 5. c. The complicated mutations of 1151 ins TTGTC + 1142A>C, del 1099-1151 +1215G>T, and del 1114-1144+del 1221 in CALR exon 9 gene showed in the melting peaks of 6, 7, and 8. The wild type of CALR exon 9 was the horizontal line in the figures a, b, and c.

Table 1. CALR* mutation types and frequencies in 21 ET# patients.

CALR exon 9 mutations (n=21)	Number (n)	Frequency (%)
del 1092-1143 (Type I)	6	28.5
1151 ins TTGTC (Type II)	7	33.3
del 1102-1137 ins CA	1	4.8
del 1099-1150	3	14.2
del 1122-1140	1	4.8
Type II + 1142A>C	1	4.8
del 1099-1151 +1215G>T	1	4.8
del 1114-1144+del 1221	1	4.8

* Calreticulin, # Essential thrombocythemia

significant difference over gender between JAK2 and CALR mutations. However, patients with CALR mutations were younger and had a higher platelet count than patients with JAK2 mutation significantly (p<0.05) (**Table 2**). Patients with JAK2 mutations had significantly higher leukocytes and hemoglobin levels and more thrombotic events than patients with CALR mutations (p<0.05).

Discussion. Besides JAK2V617F mutation, recent studies showed that somatic mutation at exon 9 of CALR accounts of 50~80% JAK2V617F-negative ET and MF patients.[5-9] It has diagnostic importance and also contributes to the clinical characteristics of ET patients; therefore, it is crucial to identify the mutations.[10-15] Although most of the mutations correspond to a 52 bp deletion (type 1) or a 5 bp insertion (type 2), there were more than 50 other mutations found. The variations of mutations continue to pose a challenge for a rapid and effective survey.[15]

The incidence of CALR mutation in our patients was similar to previous reports. However, the frequency of type 1 mutation was lower, with only half of the mutated patients presenting either type 1 or 2 mutations. The distribution was different from other reports.[7,8,10,13,14,15] Recently, Keaney and Li et al., reported a lower incidence of type 1 mutation in myelofibrosis patients.[10,12] These data might suggest that there is a population difference in CALR mutations and highlight the need for further mutation survey outside type 1 and 2.

HRM system using the saturating dsDNA binding dye for mutation survey and genotyping without the need for costly labeled oligonucleotides is an important step forward for mutation survey. In previous studies with HRM methods, it could identify type 1 and 2 mutations effectively and rapidly[13,15] were also shown in our studies. Our results were able to detect 6 other mutation types concurrently and clearly by the HRM method relating to the CALR mutations even when the mutation is outside type 1 or 2.

Our previous reports showed typical presentations of JAK2 V617F mutation patients, characterized by frequent leukocytosis and thrombosis,[2] here, the clinical presentations of the two groups with either JAK2 or CALR mutation were similar to previous reports.[5,11,14] Patients with JAK2 mutations had a higher WBC count, hemoglobin level, and thrombotic events, while patients with CALR mutations were younger and had a significantly higher platelet count. Some reports also demonstrated different clinical manifestations between type 1 and 2 CALR mutations;[10,14-15] however, due to the small number of studied cases, no differences were shown in this study.

Conclusions. The HRM method provides a fast and effective tool for the identification of clinical variants of CALR genes and the understanding of the predisposition of the disease. This report is the first on ET cases in Taiwanese cases by HRM method, and we identified six mutations that were outside type 1 or 2 mutations on CALR that may indicate a predisposition to the disease. Patients with CALR mutation had different clinical presentations when compared to JAK2 mutation and should be checked.

Table 2. Characteristics of patients with different mutations.

	Total ET* (n=60)	JAK2 (+) (n=34)	CALR# (+) (n=21)	p value (JAK2 vs. CALR)
Gender (male/female)	31/29	17/17	11/10	0.788
Age onset (years)	53.6 (18-90)	58.5 (20-90)	40.5 (18-81)	**0.017**
WBC (×1000/μl)	11.8 (4.3-26.6)	12.9 (7.2-26.6)	9.8 (4.3-19.1)	**0.040**
Haemoglobin (g/dl)	13.2 (8.3-17.6)	13.8 (9.2-17.6)	12.6 (8.4-15.4)	**0.040**
Platelets (×1000/μl)	1012.8 (631-2537)	850.3 (645-1378)	1239.4 (631-1993)	**0.030**
Thrombotic events (%)		14 (41.17)	3 (14.29)	**0.026**

Statistically significant P values are shown in bold ($p < 0.05$). *Essential thrombocythemia, #Calreticulin.

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