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Simultaneous screening for JAK2 and calreticulin gene mutations in myeloproliferative neoplasms with high resolution melting

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ABSTRACT

Introduction: Recently, novel calreticulin (CALR) mutations were discovered in Janus kinase 2 (JAK2) non-mutated myelofibrosis (PMF) and essential thrombocythemia (ET) cases, with a frequency of 60–80%. We examined clinical correlations and CALR mutation frequency in our myeloproliferative neoplasms (MPN) cases, and introduce an effective test method for use in clinical practice.
Methods: We examined 177 samples previously investigated for the JAK2 mutation for differential diagnosis of MPN. JAK2 and CALR mutations were analyzed using melting curve analysis and microchip electrophoresis, respectively. Next, we constructed a test for simultaneous screening of the JAK2 and CALR mutations utilizing high resolution melting (HRM).
Results: Among 99 MPN cases, 60 possessed the JAK2 mutation alone. Of the 39 MPN cases without the JAK2 mutation, 14 were positive for the CALR mutation, all of which were ET. Using our novel screening test for the JAK2 and CALR mutations by HRM, the concordance rate of conventional analysis with HRM was 96% for the JAK2 mutation and 95% for the CALR mutation.
Conclusion: Our novel simultaneous screening test for the JAK2 and CALR gene mutations with HRM is useful for diagnosis of MPN.

1. Introduction

Myeloproliferative neoplasms (MPN) are a group of chronic myeloid cancer diseases characterized by overproduction of mature blood cells [1,2]. Except for chronic myelogenous leukemia (CML), the 3 most common types of MPN are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). In many cases, these diseases develop slowly and gradually worsen, while some can progress to leukemia. Many patients with BCR-ABL-negative MPN carry the Janus kinase 2 V617F (JAK2) mutation [3,4]. Either that or JAK2 exon 12 mutation are found in most patients with PV, whereas one of those is found in only 50–60% of patients with ET or PMF [5–7]. In addition, mutations in the thrombopoietin receptor gene (myeloproliferative leukemia, MPL) have been reported in patients with JAK2 mutation-negative MPN. MPL mutational frequencies are estimated at 3% in ET and 10% in PMF [8]. Tests for the JAK2 mutation have greatly simplified diagnosis of MPN and are now firmly established as front-line examinations [9]. However, distinguishing ET cases possessing non-mutated JAK2 from the much more common reactive thrombocytosis remains a major diagnostic problem.

In December 2013, two groups reported novel calreticulin (CALR) mutations (exon 9 deletions and insertions) in ET and PMF patients possessing non-mutated JAK2 or MPL [10,11]. In the first of those studies [10], they examined 1107 samples from patients with MPN and noted that CALR mutations were not seen in any with PV ($n = 382$), whereas they were detected in patients with ET ($n = 311$) or PMF ($n = 203$). Interestingly, the occurrence of CALR mutations was
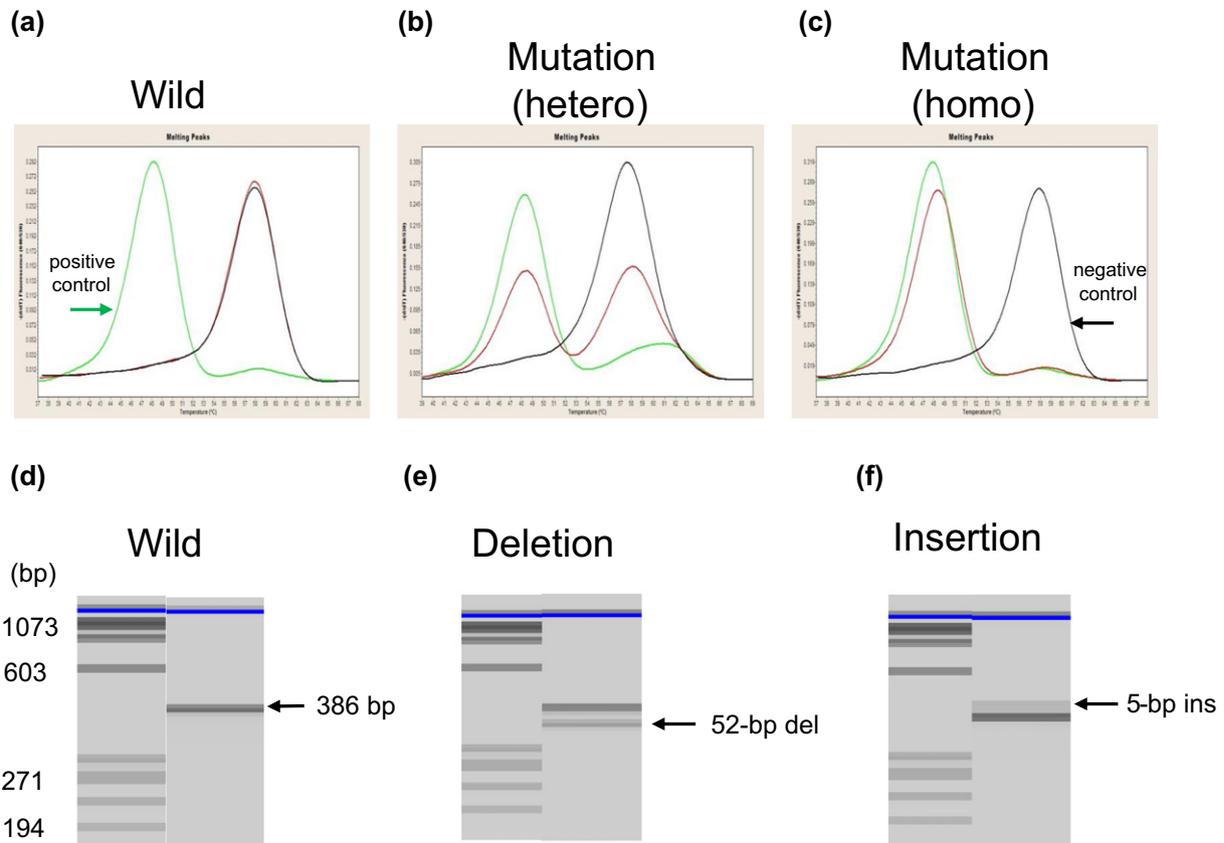
Fig. 1. Mutation analysis of JAK2 by melting curve analysis and the CALR gene by microchip electrophoresis. (a–c) Representative results of melting curve analysis for JAK2. Green line: positive control. Black line: negative control. Red line: representative samples. (a) wild-type JAK2 (b) heterozygous JAK2 mutation (c) homozygous JAK2 mutation. (d–f) Representative results of PCR for the CALR gene. (d) A single 386-bp band (arrow) was observed in specimens with the wild-type CALR gene. (e) A band smaller than that seen with the wild type was observed in the 52-bp deletion type. (f) An additional band was detected in the 5-bp insertion type.

mutually exclusive of the presence of JAK2 or MPL mutations. Accordingly, the authors examined 211 additional JAK2/MPL-non-mutated cases and found that the CALR mutational frequency in JAK2/MPL-negative disease was 67% in ET and 88% in PMF. In consideration of the promising results presented in recent reports, diagnostic criteria used for MPN may be revised in the near future. In the present study, we examined the frequency and clinical correlations of the JAK2 and CALR mutations in MPN patients treated at our institution, and introduce our novel screening testing method utilizing high resolution melting (HRM) for use in clinical practice.

Table 1
Frequencies of JAK2 and CALR gene mutations in 177 cases.

Clinical diagnosis	No. of samples	JAK2 mutation		CALR mutation	
		Occurrence	Frequency (%)	Occurrence	Frequency (%)
MPN	99				
ET	43	24	56	14	43
PV	38	32	84	0	0
PMF	7	4	57	0	0
CML	11	0	0	0	0
Secondary or stress polycythemia	15	0	0	0	0
Reactive thrombocytosis	8	0	0	0	0
MDS	5	0	0	0	0
AML	3	0	0	0	0
Others	47	0	0	0	0

2. Materials and methods

2.1. Sample preparation

We examined samples from 177 patients previously investigated for the presence of the JAK2 mutation for differential diagnosis of MPN in patients treated at Nagasaki University Hospital, between 2008 and 2014. The final clinical diagnosis was PV in 38 cases, ET in 43, PMF in 7, secondary or stress polycythemia in 15, reactive thrombocytosis in 8, CML in 11, MDS in 5, AML in 3, and others in 47. Diagnosis of PMN or other diseases was performed at the time of the first observation in accordance with the WHO criteria presented in 2008.

This retrospective study received approval from the Ethics Committee of Nagasaki University Hospital (No. 16042540), and all procedures used were in accordance with the Helsinki Declaration, revised in 2000.

2.2. Detection of JAK2 V617F mutation using melting curve analysis

DNA was extracted from bone marrow samples or peripheral blood using QuickGene-800 (FUJIFILM, Japan). Codon 12 of the JAK2 gene was amplified using the LightCycler platform (Roche, Basel, Switzerland). Real-time PCR was performed using each specimen with 5 μL of purified DNA extract in a total reaction volume of 20 μL that included 2 μL of FastStart DNA Master Hybri Probe 10× reaction master mix (Roche) under the following conditions: forward primer, 5′-AAGCAGCAAGT ATGATGAGCAA-3′ (final concentration 0.5 μM); reverse primer 5′-AGCTGTGATCCTGAAACTGAA-3′ (final concentration 0.5 μM); FITC probe 5-GTAGTTTTACTTACTCTCGTCTC-FITC-3 (final concentration
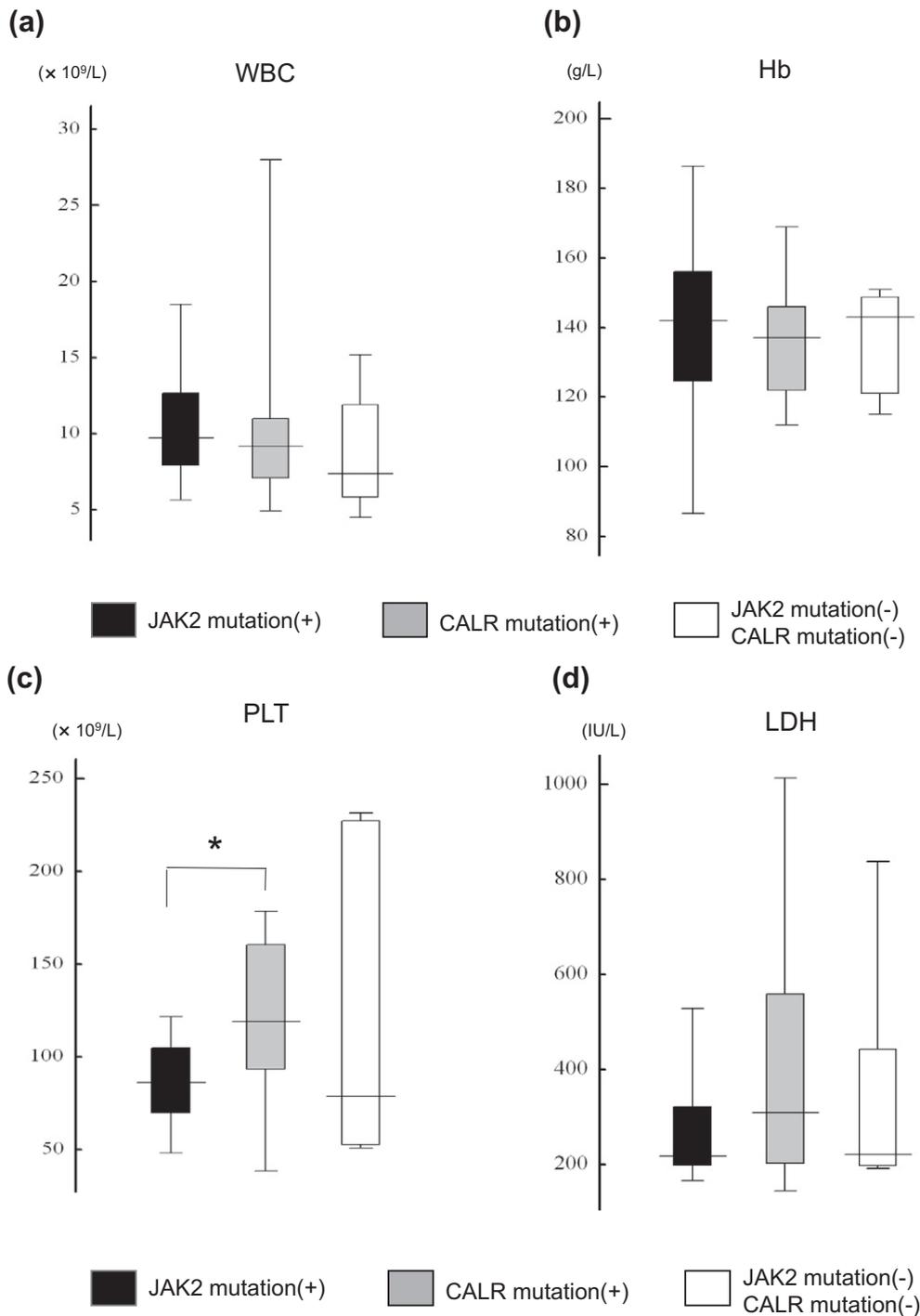
(a)

(b)

(c)

(d)

Fig. 2. Comparison of laboratory findings among ET cases possessing the CALR mutation, JAK2 mutation, or no mutation. (a, b, e) There were no significant differences among WBC count, Hb level, and MCV. (c) PLT count in cases possessing the CALR mutation was significantly higher as compared to the JAK2 mutation-positive cases. (d, f) LD level showed a tendency to be higher and MPV a tendency to be lower in cases possessing the CALR mutation as compared to the JAK2 mutation-positive cases. *P values < 0.05 were regarded to indicate statistical significance.

0.2 μM); LCRed 640 5′-LCRed640-CAGACACATACTCCATAATTT-3 (final concentration 0.2 μM), and nuclease-free water. The PCR cycle parameters were an initial denaturing step at 95 °C for 10 min and 40 cycles consisting of 95 °C for 10 s, 60 °C for 10 s, and 75 °C for 15 s. DNA melting curve analysis was performed by denaturing at 95 °C for 0 s, annealing at 35 °C for 10 s, and melting with a transition rate of 0.20 °C/s to 80 °C. Either wild-type or homozygous mutation was defined by the presence of one temperature peak and a heterozygous mutation was defined by the presence of two temperature peaks.

2.3. Detection of CALR gene mutation by microchip electrophoresis and direct sequencing

DNA was extracted from the samples and amplified by PCR using the following primers: forward, 5′-ACAACTTCCTCATCACCAACG-3′; reverse, 5′- GGCCTCAGTCCAGCCCTG-3′. Each PCR reaction was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA) under the following conditions: 35 total cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for
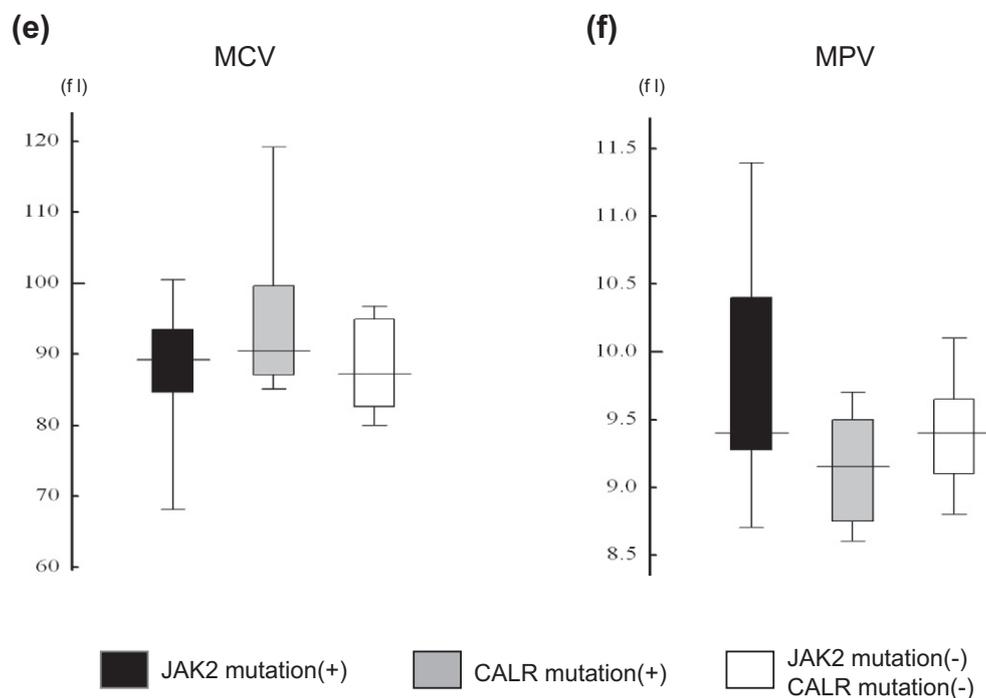
Fig. 2 (*continued*).

20 s. The amplified products were analyzed by using a MultiNA microchip electrophoresis system MCE-202 (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. To confirm the positivity of a CALR mutation, direct sequencing was performed in both directions using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

2.4. Simultaneous screening for JAK2 and CALR gene mutations with high resolution melting (HRM)

The primers used for JAK2 and CALR were as follows. JAK2: forward, 5′-GGGTTTCCTCAGAACGTTGA-3′; reverse, 5′-CTGACACCTAGCTGTGA TCCTG-3′, and CALR forward: 5′-TAACAAAGGTGAGGCCTGGT-3′; reverse, 5′-GGGACATCTTCCTCCTCATCT -3′. A LightCycler 480 (Roche) was used for HRM and real-time thermal cycling. The reactions were performed in 20 μL reaction volumes containing AmpliTaq Gold 360 Master Mix (Applied Biosystems) and GC enhancer HRM ResoLight dye. The reaction conditions for real-time PCR were as follows: 45 cycles of thermal denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The reaction conditions for HRM were 95 °C for 60 s and 40 °C for 60 s, which were determined from the fluorescence intensity of the melting curves by lowering the temperature. Analysis was performed using LightCycler 480 and the temperature shifts for JAK2 were 80.5–81 °C and 90–90.5 °C, for the low and high temperature ranges, respectively, while those for CALR were 73–73.5 °C and 79.5–80 °C, respectively. An additional HRM analysis for the CALR mutation was performed according to a protocol previously described [12].

2.5. Statistical analysis

Mann-Whitney's *U* Test was used to determine statistical significance, with *P* values <0.05 regarded as significant.

3. Results

3.1. Mutation analysis of JAK2 by melting curve analysis and the CALR gene by microchip electrophoresis and direct sequencing

We initially focused on 99 cases diagnosed as MPN and investigated the JAK2 mutation using melting curve analysis. Representative melting curves are shown in Fig. 1. A peak of wave form was observed in concordance with that of negative control in cases with the wild-type JAK2 (Fig. 1a). In those with a heterozygous or homozygous JAK2 mutation, a peak of wave form was shifted (Fig. 1b, c). The final diagnosis for these cases was PV in 38, ET in 43, PMF in 7, and CML in 11. As shown in Table 1, the JAK2 mutation was detected in 32 of the 38 PV cases (84%), 24 of the 43 ET cases (56%), and 4 of the 7 PMF cases (57%) (See Supplementary Table S1 for detailed information). Therefore, 61% (60/99 cases) of the present MPN patients had the JAK2 mutation. Next, the CALR gene mutation was analyzed using a microchip electrophoresis technique. A single band was observed in cases with the wild-type CALR gene in exon 9 (Fig. 1d, arrow). In those with a 52-bp deletion in exon 9, a band smaller than that of the wild type was detected (Fig. 1e). Similarly, in cases with a 5-bp insertion, an additional band was detected (Fig. 1f). All cases with the CALR gene mutation were confirmed by findings of sequencing analysis. As a result, 14 cases were positive for the CALR mutation. That mutation was observed only in ET cases and not detected in cases positive for JAK2 (0/60 cases). Among 39 MPN cases without the JAK2 mutation, 14 (36%) were positive for the CALR mutation and 74% of all ET cases without the JAK2 mutation were positive for the CALR mutation. None of our cases were found to have an MPL mutation. Consequently, among 43 ET cases, 38 (88%) possessed either the JAK2 or CALR mutation. In accordance with previous reports, JAK2 and CALR mutations observed in MPN patients were mutually exclusive and most instances of the CALR mutation were observed as a 52-bp deletion (type 1) or a 5-bp insertion (type 2). In the present cohort, 7 patients were deletion type and 7 were insertion type.

3.2. Clinical characteristics of ET cases with or without CALR mutation

Previous studies have found that ET patients with the CALR mutation were older, had a lower hemoglobin (Hb) level and white blood cell (WBC) count, and higher platelet (PLT) count and serum erythropoietin level as compared to those with the JAK2 mutation. In the present study, we investigated WBC count, Hb level, PLT count, mean corpuscular volume (MCV), mean platelet volume (MPV), and lactate dehydrogenase (LD) level in ET patients, and compared between those with and without the CALR and JAK2 mutations (Fig. 2). PLT count in patients possessing the CALR mutation was significantly higher as compared to JAK2 mutation-positive patients (Fig. 2c). LD level showed a tendency to be higher and MPV a tendency to be lower in patients with CALR mutation than in those with the JAK2 mutation, though the differences were not statistically significant (Fig. 2d, f). WBC count, Hb level, and MCV were not significantly different between these groups (Fig. 2a–e).

3.3. Construction of simultaneous HRM test for determining JAK2 and CALR mutations

Next, we constructed a screening test for simultaneous determination of the JAK2 and CALR gene mutations by utilizing high resolution melting (HRM). Representative HRM curves are shown in Fig. 3a and b (upper column). All 177 cases (including the above 99 cases) suspected to be MPN and subjected to JAK2 mutation analysis in our laboratory were analyzed using our novel test method (Fig. 3a and b, lower column). As a result, the cluster of wild-type cases has been clearly separated as shown in Fig. 3c and d (upper column). Similarly, in cases with heterozygous JAK2 mutation, an evident cluster was observed (Fig. 3c middle). However, in cases with homozygous JAK2 mutation, the cluster was not clear and some curves looks like that of wild-type (Fig. 3c lower column). Cases possessing mutated CALR showed rounded curves (deletion) and lopsided shaped curves (insertion) (Fig. 3d middle and lower column). After performing the simultaneous screening test for the JAK2 and CALR gene mutations with HRM, we compared the results to those obtained with conventional methods (melting curve analysis for JAK2, microchip electrophoresis for CALR) (Table. S1). The rate of concordance of conventional analysis findings with those obtained with our HRM method was 89% for the JAK2 mutation and 95% for the CALR mutation.

3.4. Additional validation of screening for JAK2 using HRM and another protocol for CALR using HRM

In the first screening step, 14 cases that showed positive for the JAK2 mutation by the conventional method were negative with our HRM method, suggesting a false negative result in each of those. Therefore, we added a wild-type spike technique to our HRM method for determining the presence of the JAK2 mutation [13]. Using mixture samples containing 40% wild-type DNA, 12 of those 14 cases successfully
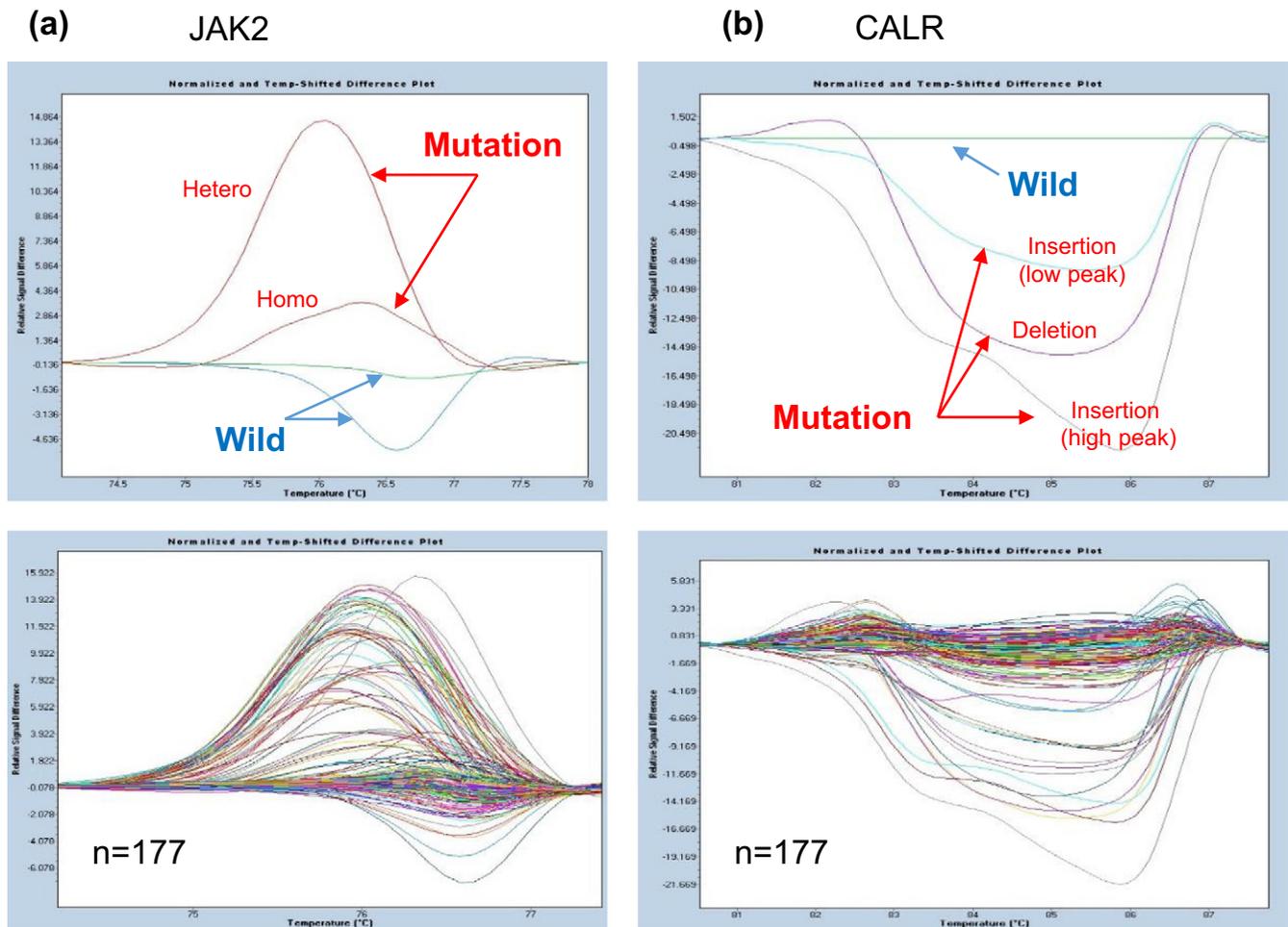

Fig. 3. Results of simultaneous screening test for JAK2 and CALR mutations in 177 cases. (a, upper) Representative HRM curves of wild-type JAK2, homozygous, and heterozygous mutations. (b, upper) Representative HRM curves of wild-type CALR, and low and high peaks of insertion and deletion type. (a,b, lower) Summarized HRM data for JAK2 and CALR mutations. (c,d, upper) The cluster of wild-type JAK2 and CALR. * a mutated sample added in order to show the whole picture. (c, middle and lower column) The cluster of heterozygous and homozygous JAK2 mutation. (d, middle and lower column) The cluster of deletion and insertion types of CALR mutation.
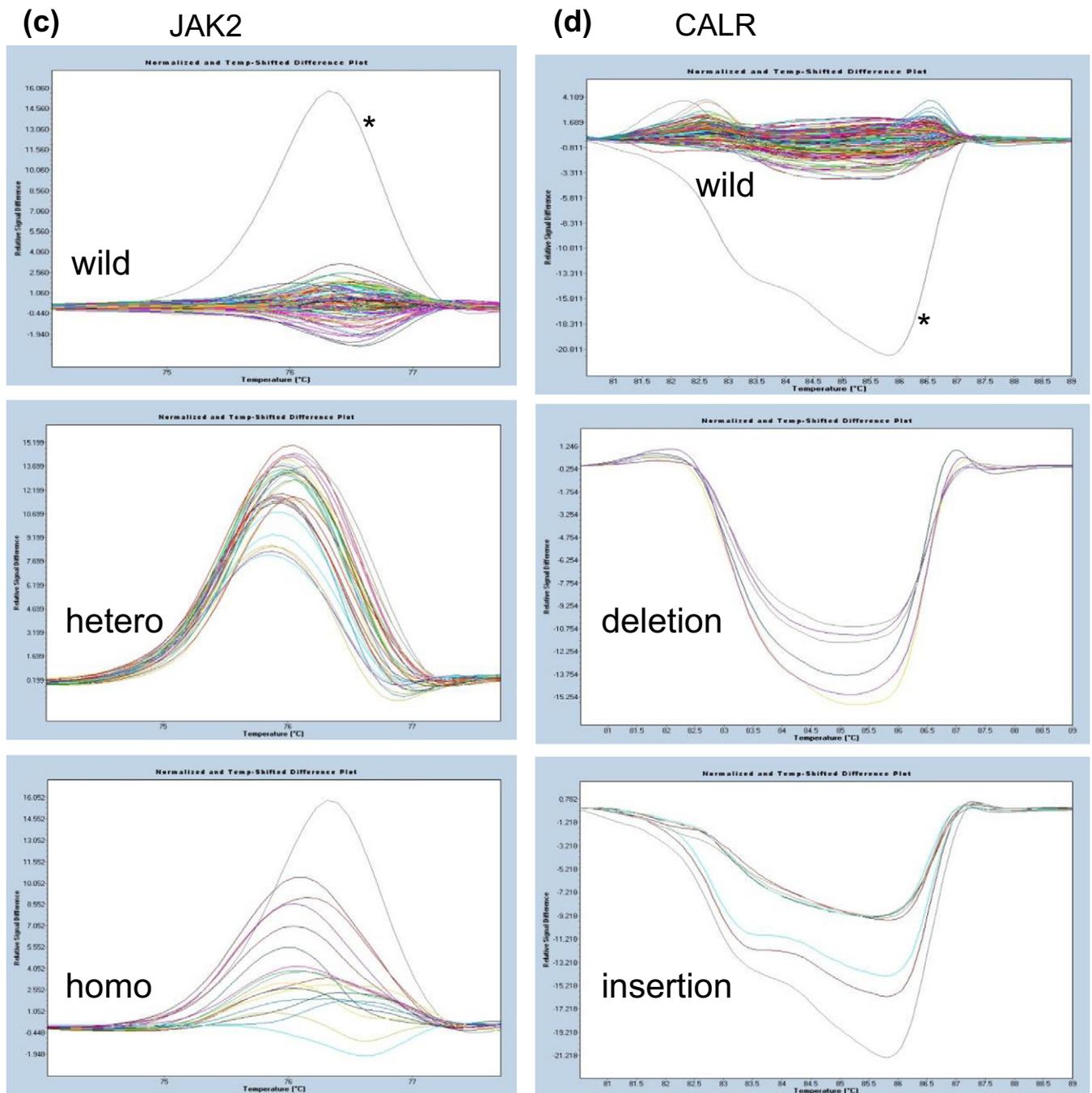
(c) JAK2

(d) CALR

Fig. 3 (*continued*).

displayed higher peaks in the HRM findings, and we were able to discriminate between specimens with the wild-type and those with the mutation (Fig. 4a, b). Although addition of JAK2 mixture samples for HRM was required in this setting, the rate of concordance was improved. As a result, the concordance rate of conventional analysis with our HRM method for the JAK2 mutation rose to 96% (Table 2).

In addition, we investigated another HRM protocol for determining the CALR mutation, as a recent report suggested that HRM for that mutation was useful and showed a good concordance rate with a conventional technique [12]. To evaluate this method in a simultaneous test setting, we examined 14 CALR mutation cases (7 insertion type, 7 deletion type) and 20 non-mutated cases. However, the concordance rate for the CALR mutation in the present cases with use of this method was only 64% (data not shown).

4. Discussion

In the present study, we determined the presence of the CALR gene mutation using microchip electrophoresis and direct sequencing results in 177 patients who were previously investigated for the presence of the JAK2 mutation as differential diagnosis of MPN at Nagasaki University Hospital. All of the CALR mutation-positive cases were observed in ET cases, and the CALR mutational frequency in JAK2/MPL-negative ET cases was 74%. Among 43 ET cases, 38 (88%) possessed either the JAK2 or CALR mutation. Klampfl, et al. reported that CALR mutational frequencies were detected in 67% and 88% of JAK2/MPL-negative ET and PMF cases, respectively. Similarly, Nangalia, et al. found the CALR mutation in 71% of patients with ET and 56% of those with PMF. Although our cohort included a small number of PMF cases, the present results in our
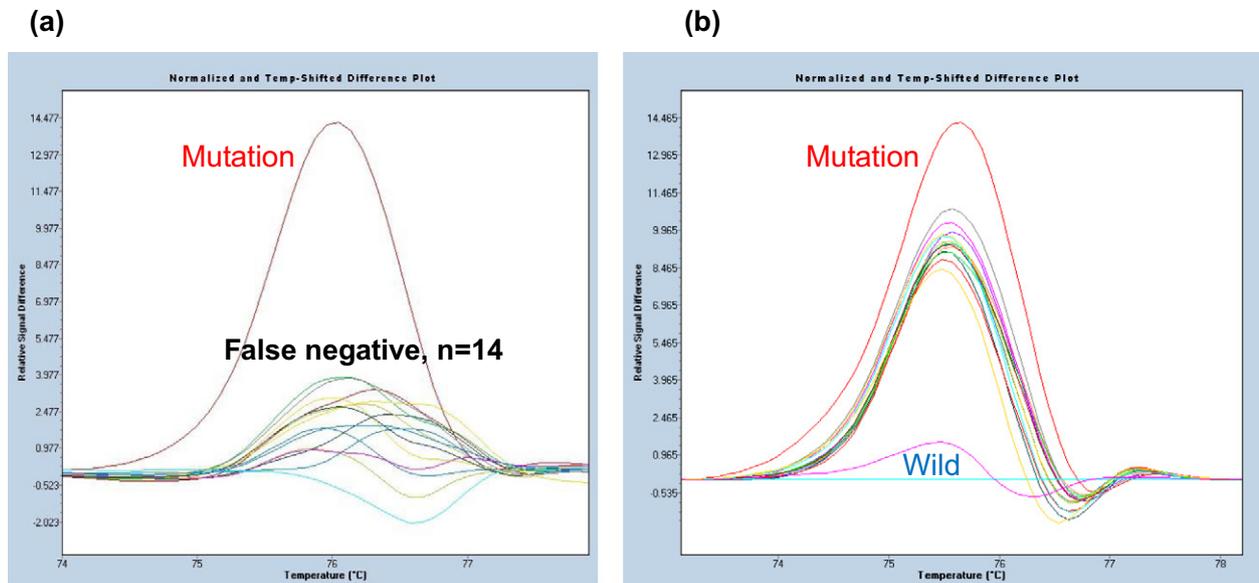
(a) **(b)**

Fig. 4. Additional validation of screening for JAK2 using HRM (a) JAK2 HRM curves of 14 cases shown positive for the JAK2 mutation by the conventional method and negative with our HRM method. These cases possessed homozygous JAK2 mutations, making them difficult to analyze using HRM. (b) Wild-type spike technique for HRM method to determine presence of the JAK2 mutation. Of the 14 false-negative cases in Fig. 4a, higher peaks were successfully displayed in 12 using the wild-type spike technique, allowing for discrimination between the specimens possessing the wild-type and the mutation.

ET patients were in accordance with previous reports and indicate that the frequency of the CALR mutation in Japan is similar to that in western populations. Considering the promising results previously reported as well as our present findings, we concluded that determination of the CALR mutation is very useful for diagnosis of MPN and indicate the need for revision of the diagnostic criteria in the near future.

Previous reports have found that ET patients with CALR mutation are older, have a lower Hb level and WBC count, and higher PLT count and serum level of erythropoietin as compared to those with the JAK2 mutation. Our results also showed that the PLT count in cases with the CALR mutation was significantly higher as compared to JAK2 mutation-positive cases. In addition, LD level showed a tendency to be higher in our cases with the CALR mutation as compared to those possessing the JAK2 mutation. These results may suggest that cells with the CALR mutation have different proliferative mechanisms as compared to those possessing the JAK2 mutation.

Although the significance and function of the CALR gene mutation have not fully investigated, Klampf, et al. presented interesting experimental results. They constructed cells expressing a type 1 CALR mutation (52-bp deletion) using the interleukin-3 (IL-3)-dependent murine cell line Ba/F3, which shows growth independent of IL-3 as well as hypersensitivity to IL-3. To investigate whether IL-3 independence in the CALR type 1-mutated cells was caused by activation of JAK-signal transducer and activator of transcription (JAK-STAT) signaling, they investigated their sensitivity to SAR302503, a JAK2 kinase inhibitor. Cells expressing non-mutated CALR or the type 1 CALR mutation showed sensitivity to SAR302503, suggesting that IL-3-independent growth of cells possessing mutated CALR is depend on JAK2 or a JAK family kinase targeted by SAR302503. Their results suggest that the CALR mutation has a relationship with tumor growth activity, in part due to the JAK-STAT signaling pathway.

For the present study, we constructed a novel test for simultaneous screening of the JAK2 and CALR gene mutations by utilizing HRM, as a recent study showed the possibility of using HRM for CALR and reported good rates of concordance with conventional methods [12]. Based on these results, we modified the protocol for testing CALR with HRM. Unfortunately, the results were not adequate, as the concordance rate in our experiment was only 64%, though we are not able to explain why the results did not fully match. As for our method for using HRM to determine possession of the JAK2 mutation, we added a single spike technique in order to avoid false negative findings, which may have been caused at least in part by automated protocol used by the LightCycler 480 device. When samples have homozygous JAK2 mutations, the waveform peak shows a low curve, making it difficult to distinguish the wild from the mutant-type. Among the various mixing patterns observed with samples possessing wild-type DNA, mixture samples containing 40% of the wild-type were superior for discrimination between wild and mutation-type samples. Although addition of JAK2 mixture samples is required in this setting and there is room for further improvement, this technique was also effective against the unknown samples in our observation. As a result, the concordance rate of the conventional method and our simultaneous HRM method for the JAK2 mutation was 96% and that for CALR mutation was 95%, which we consider to be adequate for a screening test.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.09.023.

Table 2
Rate of concordance of conventional analysis with present HRM method.

		JAK2 mutation		CALR mutation	
		Melting curve analysis		Microchip electrophoresis	
		Positive	Negative	Positive	Negative
HRM	Positive	60	6	14	0
	Negative	2	109	9	154
	Match	169		168	
	Mismatch	8		9	
Concordance ratio (%)		96		95	

HRM: High resolution melting.

Authors' contributions

N.M. performed HRM experiments and wrote the manuscript.
S.M. designed PCR experiments and examined clinical data.
H.H. designed this study and wrote the manuscript.
H.M. performed PCR experiments.
D.S., and K.T. designed PCR and HRM experiments.

D.I., Y.I., and T.H. contributed to preparation of clinical samples.

N.U., K.K., and N.K. analyzed results and critically read the manuscript.

Y.M. arranged preparation of clinical samples and commented on the manuscript.

Y.K. arranged funding, supervised experiments and mentored all work.

Conflicts of interest disclosure

The authors declare no conflict of interest associated with this manuscript.

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