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Screening PCR Versus Sanger Sequencing: Detection of *CALR* Mutations in Patients With Thrombocytosis

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Background: Mutations in calreticulin (*CALR*) have been reported to be key markers in the molecular diagnosis of myeloid proliferative neoplasms. In most previous reports, *CALR* mutations were analyzed by using Sanger sequencing. Here, we report a new, rapid, and convenient system for screening *CALR* mutations without sequencing.

Methods: Eighty-three bone marrow samples were obtained from 81 patients with thrombocytosis. PCR primers were designed to detect wild-type *CALR* (product: 357 bp) and *CALR* with type 1 (product: 302 bp) and type 2 mutations (product: 272 bp) in one reaction. The results were confirmed by Sanger sequencing and compared with results from fragment analysis.

Results: The minimum detection limit of the screening PCR was 10 ng for type 1, 1 ng for type 2, and 0.1 ng for cases with both mutations. *CALR* type 1 and type 2 mutants were detected with screening PCR with a maximal analytical sensitivity of 3.2% and <0.8%, respectively. The screening PCR detected 94.1% (16/17) of mutation cases and showed concordant results with sequencing in the cases of type 1 and type 2 mutations. Sanger sequencing identified one novel mutation (c.1123_1132delinsTGC). Compared with sequencing, the screening PCR showed 94.1% sensitivity, 100.0% specificity, 100.0% positive predictive value, and 98.5% negative predictive value. Compared with fragment analysis, the screening PCR presented 88.9% sensitivity and 100.0% specificity.

Conclusions: This screening PCR is a rapid, sensitive, and cost-effective method for the detection of major *CALR* mutations.

Key Words: *CALR*, Screening PCR, Sanger sequencing, Fragment analysis

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INTRODUCTION

The classic Philadelphia chromosome-negative (Ph$^-$) myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). In 2005, Janus kinase 2 (*JAK2*) V617F mutations were detected in 95%, 50%, and 60% of PV, ET, and PMF patients, respectively [1]. A few patients carry other *JAK2* mutations, such as insertions or deletions in exon 12 or mutations in the thrombopoietin receptor (*MPL*). Approximately 40% of ET and PMF patients lack a reliable genetic marker of disease [2]. At the end of 2013, recurrent mutations in the calreticulin (*CALR*) gene were identified in two studies using whole-exome sequencing [3, 4]. These studies identified recurrent mutations in *CALR* in 60-88% of patients with ET and PMF who were negative for *JAK2* and *MPL* mutations. *CALR* mutations were not found in healthy control subjects or in cases of lymphoid neoplasia, acute leukemia, or solid tumors, indicating specificity for ET and PMF [3, 4]. All *CALR* mutations are insertions or deletions in exon 9, and the most common mutations, accounting

for 80-90% of mutation cases, were either type 1, a 52-bp deletion (c.1092_1143del; p.L367fs*46), or type 2, a 5-bp insertion (c.1154_1155insTTGTC, pK385fs*47). Other infrequent mutations in exon 9 account for up to 15% of *CALR* mutations [2]. All known recurrent *CALR* mutations lead to a frameshift that generates a common 36 amino acid C-terminal end, and to the loss of the KDEL motif. The distribution of *CALR* mutation types differs according to the MPN type [5].

In most previous reports, *CALR* mutations were analyzed by Sanger sequencing [3, 4, 6, 7]. However, this sequencing method is time-consuming and cannot be performed in every laboratory. Here, we present a convenient system for screening major *CALR* mutations that does not require sequencing analysis. We investigated the efficiency of the screening PCR to detect *CALR* mutations in the Korean patients with thrombocytosis and compared the results with those from Sanger sequencing, the reference method, and fragment analysis, a sensitive detection method.

METHODS

1. Patient selection

Eighty-one patients with thrombocytosis who underwent a bone marrow (BM) study at Gachon University Gil Medical Center in Korea from April 2007 to February 2015 were enrolled in this retrospective study. BM samples were obtained from patients at diagnosis (81 samples) or in a follow-up visit (2 samples). The patients included 54 with ET; three with PMF; three with myelodysplastic neoplasms (MDNs) or MPNs, unclassifiable (MDS/MPN, U); two with MPNs, unclassifiable (MPN, U); one with hypereosinophilic syndrome (HES); and 20 that were benign disease cases. The clinical and laboratory data were obtained from medical records. This study was approved by our institutional review board (GCIRB2015-73), and informed consent was obtained from all enrolled patients at that time of BM study.

2. Patient characteristics

We compared the laboratory data of patients according to disease and *CALR* mutation status. Statistical differences in age, sex, hemoglobin levels, red cell distribution width (RDW), white blood cell count, percent segmented neutrophils, percent eosinophils, platelet count, mean platelet volume (MPV), platelet distribution width (PDW), and lactose dehydrogenase (LDH) level were observed among the patient groups. These results are shown in Table 1.

3. Detection of *JAK2* V617F by PCR

DNA from the BM aspirates was isolated by using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). A Seeplex *JAK2* Genotyping kit (Seegene, Seoul, Korea) or Real-Q *JAK2* V617F Detection kit (BioSewoom, Seoul, Korea) was used to detect *JAK2* V617F mutations.

4. Screening PCR

For analysis of *CALR* mutations, oligonucleotide primers targeting exon 9 of *CALR* were used to amplify the mutation hot spot. PCR primer sets were designed to detect type 1 and type 2 mutations in one reaction, including primers F1 (forward primer 1) 5´-GCA GCA GAG AAA CAA ATG AAG G-3´, F2 (forward primer 2) 5´-GCA GAG GAC AAT TGT CGG A-3´, and R (reverse primer) 5´-AGA GTG GAG GAG GGG AAC AA-3´ (Fig. 1A, B). Ten nanograms of DNA template, 0.5 µL (10 pmol) of each forward primer, and 1.0 µL (10 pmol) of the reverse primer were added to the PCR premix (20 µL, final volume) (Bioneer, Daejeon, Korea). An initial preheating at 94°C for 10 min was followed by denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec, and extension at 72°C for 30 sec for 40 cycles followed by a final extension at 72°C for 7 min. After PCR amplification, gel electrophoresis was performed in a 2% agarose gel at 130 V for 30 min to detect the amplified regions of DNA, and agarose gels were exposed under UV light in a Bio-Rad Gel DOC EZ imager (Bio-Rad, Hercules, CA, USA) (Fig. 1C). Interpretation was done by comparing bands to the expected product size (wild type *CALR*: 357 bp, *CALR* type 1 mutation: 302 bp, and *CALR* type 2 mutation: 272 bp).

5. Sensitivity of screening PCR in detecting *CALR* type 1 and type 2 mutations

To study the limit of detection (LoD) of the screening PCR, patient BM DNA carrying *CALR* type 1 (No. 48) and type 2 (No. 45) were serially diluted in different concentrations (100 ng, 10 ng, 1 ng, and 0.1 ng). In the case of both mutations (No. 62), three concentrations were tested (10 ng, 1 ng, and 0.1 ng) owing to lack of sample quantity. To determine the assay sensitivity according to tumor burden, the reference sequences for wild-type *CALR* and type 1 and type 2 *CALR* mutants were obtained by gel extraction (GeneAll, Seoul, Korea) from PCR products. The weight of DNA to copy number was calculated (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr), and the samples were diluted to the same copy number with different tumor burdens (50% mutant, 25% mutant, 12.5% mutant, 6.3% mutant, 3.2% mutant, 1.6% mutant, and 0.8% mutant).

Type 1 mixtures were analyzed by Sanger sequencing for a comparison of assay sensitivity.

6. Sanger sequencing

Ten nanograms of DNA template and 1.0 μL (10 pmol) each of primers F1 and R were added to the PCR premix (20 μL final volume) (Bioneer). PCR was performed according to the conditions described above, and then PCR products were purified and sequenced by using the reverse primer and a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3500XL Genetic Analyzer (Applied Biosystems).

7. Fragment analysis

For the fragment analysis, PCR was carried out with 6-FAM-labeled F1 and R primers. PCR products were analyzed by capillary electrophoresis on an ABI 3500XL Genetic Analyzer (Applied Biosystems), followed by fragment analysis using GeneMapper Software 4.1 (Applied Biosystems).

8. Statistical analysis

Sensitivity, specificity, positive predictive value and negative predictive value were calculated by MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php). Kruskal–Wallis test was used for the analysis of continuous variables, and Fisher's exact test was used for the analysis of categorical variables. $P<0.05$ was considered statistically significant. Agreement between results of the detection methods was assessed by using the Kappa statistic (Cohen's kappa coefficient [κ]: <0=poor, 0-0.2=slight, 0.21-0.4=fair, 0.41-0.6=moderate, 0.61-0.8=substantial, and 0.81-1=almost perfect) [8]. The SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical program was used for all calculations.

RESULTS

1. Comparison of laboratory findings and *CALR* mutation status in patients with hematologic malignancies and reactive condition

Among the patients with thrombocytosis, *CALR* mutations were detected in 17 samples by Sanger sequencing; however,

Table 1. Characteristics of patients with or without *CALR* mutations in the malignancy group and benign group

	Hematologic malignancy		Reactive	P*
	With *CALR* mutation (N=17)	Without *CALR* mutation (N=46)	Without *CALR* mutation (N=20)	
Age (yr), median (1Q, 3Q)	51.0 (33.5-60.0)	64.5 (48.8-78.5)	49.5 (32.3-67.3)	0.009
Male, N (%)	8 (47.1)	26 (56.5)	4 (20.0)	0.021
Hb (g/dL), median (1Q, 3Q)	12.7 (10.4-14.3)	13.4 (10.7-15.2)	11.4 (9.5-12.6)	0.02
MCV, median (1Q, 3Q)	88.9 (85.3-91.3)	86.8 (79.7-91.2)	88.4 (83.4-91.1)	NS
MCH, median (1Q, 3Q)	28.9 (28.0-30.3)	28.5 (25.6-30.1)	29.3 (27.2-29.9)	NS
MCHC, median (1Q, 3Q)	33.0 (32.4-33.3)	32.5 (31.6-33.7)	32.8 (32.1-33.4)	NS
RDW, median (1Q, 3Q)	13.9 (13.4-15.0)	15.2 (14.2-17.7)	15.0 (13.6-16.3)	0.017
WBC, $\times 10^6$/L, median (1Q, 3Q)	8,440 (6,565-10,805)	10,565 (9,110-18,043)	9,870 (7,730-12,320)	0.009
Seg (%), median (1Q, 3Q)	64.0 (57.5-72.0)	70.5 (60.8-81.0)	63.2 (55.8-71.0)	0.039
Eosi (%), median (1Q, 3Q)	2.0 (0.5-3.3)	4.0 (2.0-5.8)	2.0 (0.3-4.0)	0.014
Baso (%), median (1Q, 3Q)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.0 (0.0-1.0)	NS
PLT, $\times 10^9$/L, median (1Q, 3Q)	1,195 (810-1,548)	791 (604-1,085)	719 (545-939)	0.018
MPV, median (1Q, 3Q)	8.5 (8.2-9.5)	9.1 (8.4-10.0)	8.1 (7.7-8.9)	0.002
PDW, median (1Q, 3Q)	46.6 (38.6-55.1)	49.8 (41.0-56.0)	39.0 (35.5-45.5)	0.012
LDH, median (1Q, 3Q)	619 (596-827)	700 (597-853)	429 (324-483)	<0.001
Eosi (%) in BM, median (1Q, 3Q)	2.6 (1.7-5.7)	3.0 (2.0-5.1)	2.2 (1.3-3.2)	NS
Baso (%) in BM, median (1Q, 3Q)	0.3 (0.0-0.4)	0.2 (0.0-0.6)	0.0 (0.0-0.4)	NS

*Kruskal Wallis test was used for continuous values and Fisher's exact test was used for categorical values.
Abbreviations: Q, quartile; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, white blood cells; Seg, segmented neutrophils; Eosi, eosinophils; Baso, basophils; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; LDH, lactose dehydrogenase; BM, bone marrow; NS, not significant.

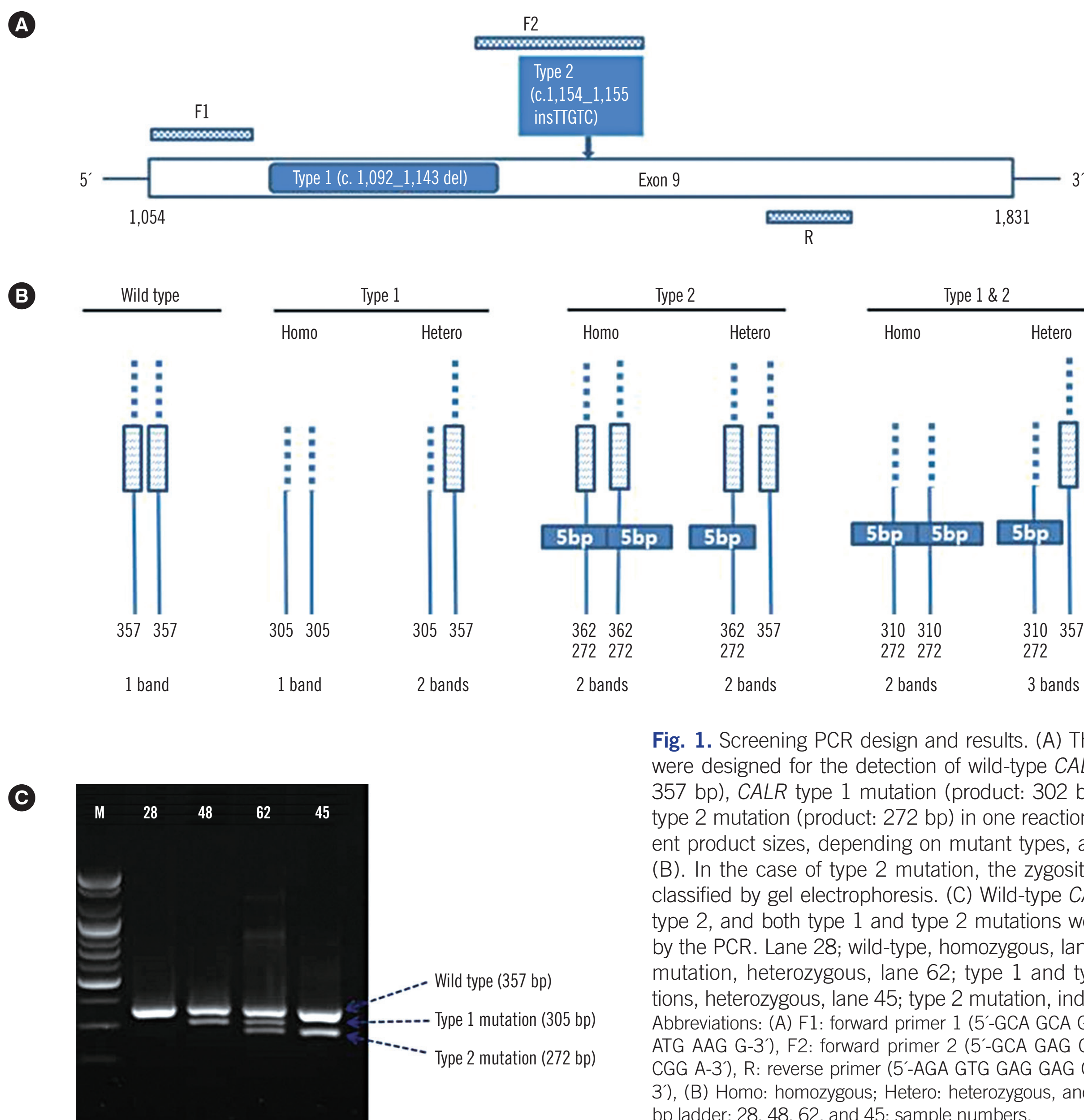

Fig. 1. Screening PCR design and results. (A) Three primers were designed for the detection of wild-type *CALR* (product: 357 bp), *CALR* type 1 mutation (product: 302 bp), or *CALR* type 2 mutation (product: 272 bp) in one reaction. The different product sizes, depending on mutant types, are shown in (B). In the case of type 2 mutation, the zygosity cannot be classified by gel electrophoresis. (C) Wild-type *CALR*, type 1, type 2, and both type 1 and type 2 mutations were detected by the PCR. Lane 28; wild-type, homozygous, lane 48; type 1 mutation, heterozygous, lane 62; type 1 and type 2 mutations, heterozygous, lane 45; type 2 mutation, indeterminate.
Abbreviations: (A) F1: forward primer 1 (5´-GCA GCA GAG AAA CAA ATG AAG G-3´), F2: forward primer 2 (5´-GCA GAG GAC AAT TGT CGG A-3´), R: reverse primer (5´-AGA GTG GAG GAG GGG AAC AA-3´), (B) Homo: homozygous; Hetero: heterozygous, and (C) M: 100-bp ladder; 28, 48, 62, and 45: sample numbers.

no *CALR* mutations were detected in patients with reactive condition.

2. Sensitivity of screening PCR in detecting *CALR* type 1 and type 2 mutations

The LoD of the multiplex screening PCR was estimated by determining the minimal genomic DNA required for generation of all of the expected bands. We first evaluated the ability of the screening PCR to detect each mutation type with different quantities of template in three patient samples (type 1: No. 48; type 2: No. 45; and both types: No. 62). The minimal quantity of template genomic DNA required for the reaction was approximately 10 ng to detect type 1 mutation, 1 ng for type 2 mutation, and <0.1 ng for both mutations (Fig. 2A). The sensitivity of the screening PCR was estimated by the minimal tumor burden required for all of the expected bands to be present. The screening PCR detected *CALR* type 1 and type 2 mutants with a maximal sensitivity of 3.2% and <0.8%, respectively (Fig. 2B), whereas the sensitivity of Sanger sequencing in detecting type 1 mutation was 6.3% (Fig. 2C). All sensitivity tests were performed with duplicate samples.

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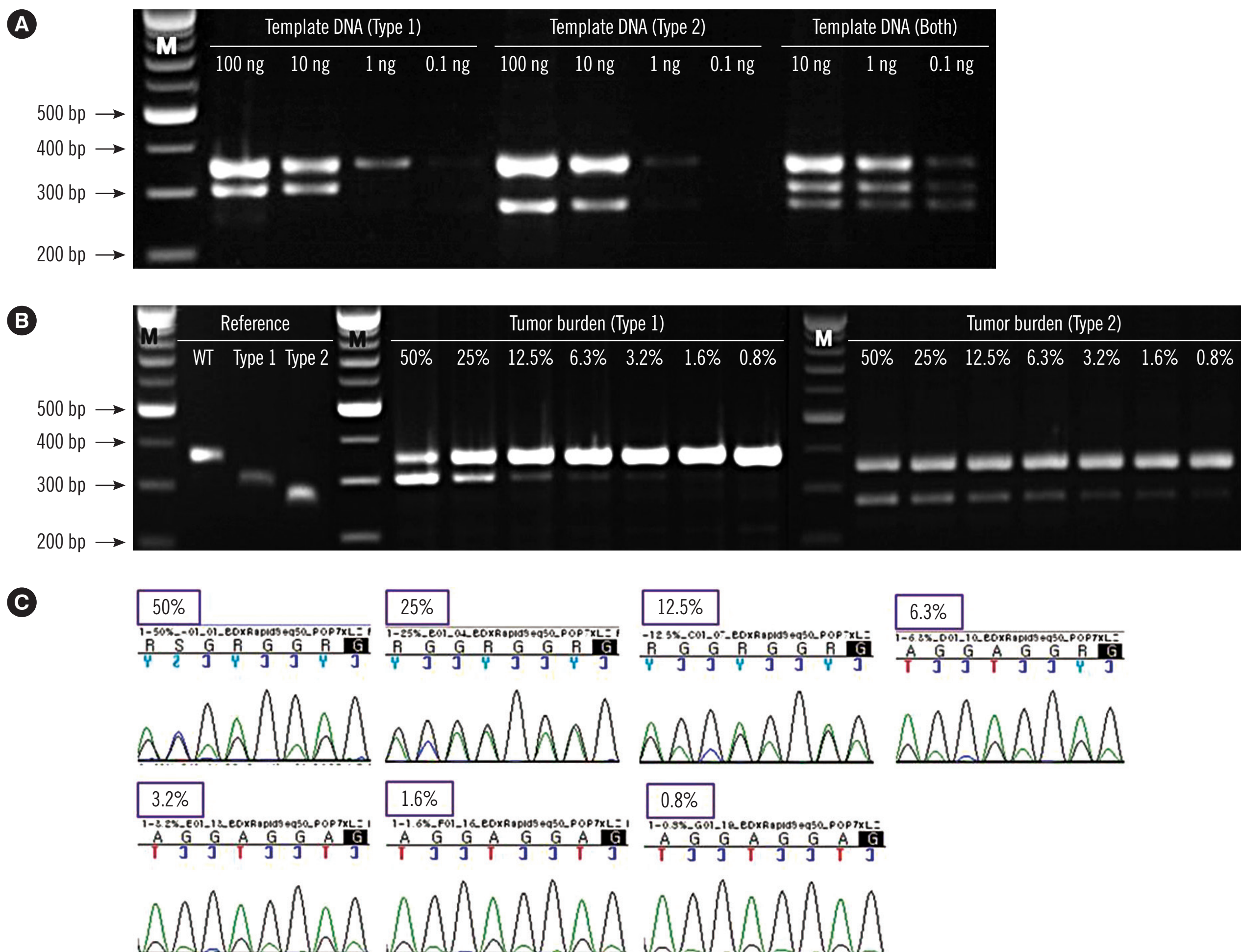

Fig. 2. Sensitivity of screening PCR in the detection of *CALR* mutations. (A) The minimal quantity of genomic DNA required for the reaction was approximately 10 ng for the type 1 mutation, 1 ng for the type 2 mutation, and <0.1 ng for both mutations. (B) Sensitivities in cases with different tumor burdens (50% mutant, 25% mutant, 12.5% mutant, 6.3% mutant, 3.2% mutant, 1.6% mutant, and 0.8% mutant) were determined by using reference sequences. Screening PCR can detect *CALR* type 1 and type 2 mutants with a maximal sensitivity of 3.2% and <0.8%, respectively, (C) compared with 6.3% or higher sensitivity by Sanger sequencing.

3. Comparison of Sanger sequencing, screening PCR, and fragment analysis for mutation detection

With Sanger sequencing, the three types of *CALR* mutations, type 1, type 2, and a novel mutation (c.1123_1132delinsTGC), were detected in 16 ET patients and one PMF patient. Type 2 mutation was the most common (9/17, 53.0%), followed by the type 1 mutation (6/17, 35.3%), and then type 1 and type 2 (1/17, 5.9%) and the novel mutation (1/17, 5.9%). The screening PCR resulted in products of different sizes depending on the mutation type (Fig. 1C). The screening PCR detected 94.1% (16/17) of mutations, and this method showed results concordant with Sanger sequencing in the cases of type 1 and type 2 mutations. However, one novel mutation was not detected in the screening PCR. Two patients (No. 32 and No. 74) showed discrepant results between the fragment analysis and the sequencing analysis and screening PCR. These samples had very low type 1 mutation burdens (0.3% and 1.6%) (Table 2). After 10-fold dilution of the PCR products from the patients No. 32 and No. 74, nested PCR was performed with same primers, and type 1 mutations were detected in the second PCR products. Almost perfect agreement between the screening PCR and the other methods was observed for *CALR* mutation detection (κ=0.962, Sanger sequencing and κ=0.926, fragment analysis) (Table 3). The screening PCR, relative to sequencing, showed 94.1% sensitivity (95% confidence intervals [CI]: 71.3-99.9%), 100.0% specificity (95% CI: 94.6-100.0%), 100.0% positive predictive value (95% CI: 79.4-100.0%), and 98.5% negative predictive value (95% CI: 92.0-100.0%). The screen-

Table 2. Mutation types detected by different methods and mutant burden in the fragment analysis

No.	Mutation type (0=WT, 1=type 1, 2=type 2, 3=both, 4=new)			Mutant burden in fragment analysis (%)*		
	Sanger sequencing	Screening PCR	Fragment analysis	Mutant	Type 1	Type 2
32†	0	0	1	0.3	0.3	0.0
29	1	1	1	49.4	49.4	0.0
37	1	1	1	25.0	25.0	0.0
48	1	1	1	51.5	51.5	0.0
67	1	1	1	50.6	50.6	0.0
76	1	1	1	31.1	31.1	0.0
92	1	1	1	22.2	22.2	0.0
131	1	1	1	43.6	43.6	0.0
74†	2	2	3	46.6	1.6	45.0
45	2	2	2	56.8	0.0	56.8
69	2	2	2	2.7	0.0	2.7
80	2	2	2	38.7	0.0	38.7
81	2	2	2	41.8	0.0	41.8
108	2	2	2	44.2	0.0	44.2
113	2	2	2	47.3	0.0	47.3
128	2	2	2	49.4	0.0	49.4
62†	3	0	3	56.7	34.4	22.3
75	4	4	4	44.1	0.0	0.0

CALR mutant burden was calculated by fragment analysis, allowing a determination of the peak area ratio between the mutant and wild-type alleles; †Sample with discrepant results between mutation detection methods.
Abbreviation: WT, wild type.

ing PCR showed 88.9% sensitivity (95% CI: 65.3-98.6%), 100.0% specificity (94.5-100.0%), 100.0% positive predictive value (95% CI: 79.4-100.0%), and 97.0% negative predictive value (95% CI: 89.6-99.6%) compared with the fragment analysis results. All mutations, including the novel mutation, resulted in a common +1 bp altered reading frame and induced a novel C-terminal peptide sequence lacking the KDEL motif.

4. Frequency of mutations and clinical characteristics in different patient groups

By sequencing, *CALR* mutations in malignancy group were detected in 17 samples (17/63, 27.0%). The *CALR* mutational frequencies in each disease subtype were 29.6% (16/54) for ET and 33.3% (1/3) for PMF (Table 4). In patients without *JAK2* V617F mutations, the *CALR* mutational frequency was 61.9% (13/21) for ET and 50% (1/2) for PMF. Three ET patients had

Table 3. Comparison of mutation detection methods, including screening PCR, Sanger sequencing, and fragment analysis

Mutation detection by screening PCR	N of detected mutations by Sanger sequencing		N of detected mutations by fragment analysis	
	Positive	Negative	Positive	Negative
Positive	16	0	16	0
Negative	1	66	2	65
Kappa value	0.962		0.926	

both *CALR* and *JAK2* V617F mutations. The prevalence of dual negative (negative for both *CALR* and *JAK2* V617F mutations) patients was 14.8% (8/54) in ET and 33.3% (1/3) for PMF patients.

In the ET group, mutational frequencies were 61.1% for *JAK2* V617F, 29.6% for *CALR*, 5.6% for dual positive, and 14.8% for dual negative mutations. Among ET patients without *JAK2* V617F, the frequency of *CALR* mutation was 61.9% (13/21). The frequency of type 2 *CALR* mutation (8/16, 50.0%) was higher than that of type 1 mutation (6/16, 37.5%). Interestingly, one patient with both mutations (1/16, 6.3%) was identified, and the novel mutation (c.1123_1132delinsTGC) was detected only by Sanger sequencing. No *CALR* or *JAK2* V617F mutation was detected in the reactive disease group. Laboratory findings in patients with *CALR* mutations are summarized in Table 5.

5. Follow-up samples

Follow-up samples from two patients were included in the study. One patient had shown mild thrombocytopenia since 2002, and the first BM study was performed in April 2007. A BM biopsy showed normal cellularity with increased megakaryocytes. Reactive disease could not be ruled out, but the first BM study did not test for the *JAK2* V617F mutation. Anagrelide (2 mg/day) was given because the thrombocytosis persisted even after using hydroxyurea. Another BM study was conducted in November 2011, and the *JAK2* V617F mutation was detected; however, no *CALR* mutations were detected. At that time, the first BM sample was tested for *JAK2* V617F and *CALR* mutations, and the *JAK2* V617F mutation was detected.

Another patient (No. 80) was 20 weeks pregnant at the time of sample collection. Thrombocytosis was detected during a regular checkup, and a BM study was performed. The BM was normocellular with an elevated megakaryocyte count. The *JAK2* V617F mutation was not found. Even though the physician highly suspected ET, the patient refused BM suppression therapy and took 100 mg aspirin a day. In March 2012, another BM study was performed because of persistent thrombocytosis after

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Table 4. Frequency of *JAK2* and *CALR* mutations in different patient groups

	ET N=54	PMF N=3	MDS/MPN, U N=3	MPN, U N=2	HES N=1	Reactive N=20	Total N=83
JAK2 mutation, N (%)	33 (61.1%)	1 (33.3%)	1 (33.3%)	1 (50.0%)	0 (0.0%)	0 (0.0%)	36 (43.4%)
CALR mutation, N (%)*	16 (29.6%)†	1 (33.3%)‡	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	17 (20.5%)
Dual positive, N (%)	3 (5.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (3.6%)
Dual negative, N (%)	8 (14.8%)	1 (33.3%)	2 (66.7%)	1 (50.0%)	1 (100.0%)	20 (100.0%)	32 (38.6%)
CALR mutation (%) in *JAK2*(-) patients	61.9%	50%					

*Result by Sanger sequencing; †Type 1 mutation: 6 patients; Type 2 mutation: 8 patients; Type 1 and Type 2 mutations: 1 patient; and novel mutation: 1 patient; ‡Type 2 mutation.
Abbreviations: ET, essential thrombocythemia; PMF, primary myelofibrosis; MDS/MPN, U, myelodysplastic syndrome/myeloproliferative neoplasm, unclassifiable; MPN, U, myeloproliferative neoplasm, unclassifiable; HES, hypereosinophilic syndrome; Reactive, reactive disease group with non-hematologic malignancy.

Table 5. Laboratory findings in patients with *CALR* mutation

CALR mutation*	No.	Dx	Age	Sex	*JAK2* V617F	LDH	Hb	MCV	MCH	MCHC	RDW	WBC	Eosinophilia	Basophilia	PLT	MPV	PDW	Mega count	Mega cluster	BM Bx cellularity	Karyotype
Type 1 (N=6)	29	ET	61	F	(-)	609	10.5	86.2	27.9	32.4	13.7	5,480	(-)	(-)	1,012,000	10.4	12.1	↑	(-)	Hyper	46,XX [20]
	37	ET	56	M	(-)	409	14.6	89.2	31.4	35.2	13.1	9,440	(-)	(+)	774,000	8.5	38	↑	(+)	Normal	46,XY [20]
	48	ET	51	M	(-)	700	13.3	90.3	29.7	32.9	14.5	7,030	(-)	(-)	654,000	11.7	55.2	↑	(+)	Hyper	46,XY [20]
	67	ET	83	F	(-)	1,192	8.8	86	26.5	30.8	17.3	10,590	(-)	(+)	1,544,000	9.2	59.1	↑	(+)	Hyper	46,XX [16]
	76	ET	44	M	(+)†	827	14.6	87	28.9	33.3	13.7	12,780	(+)	(+)	1,298,000	8.9	53	↑	(+)	Normal	46,XY [5]
	92	ET	33	F	(+)†	1,021	13.1	84.4	28.1	33.3	15.4	6,650	(-)	(-)	611,000	8.5	56.3	↑	(+)	Normal	46,XX [4]
Type 2 (N=8)	45	ET	51	F	(-)	558	10.2	84.6	28.2	33.3	14.4	4,430	(-)	(-)	1,414,000	10.2	37	↑	(-)	Normal	46,XX [20]
	69	ET	55	M	(-)	407	14.1	90.7	30.3	33.5	13.8	7,190	(-)	(-)	845,000	8.4	55	↑	(+)	Hypo	46,XY,inv (9) (p12q13) [15]
	74	ET	52	M	(-)	602	14.4	91.8	29.9	32.6	13.7	13,110	(-)	(+)	1,658,000	8.1	43	↑	(+)	Normal	46,XY [20]
	80	ET	27	F	(+)†	NT	11.7	88.9	28.1	31.6	14.3	8,440	(-)	(+)	1,911,000	8.4	39.2	↑	(+)	Normal	46,XX [20]
	81	ET	59	F	(-)	NT	12.6	93.2	31	33.3	12.3	4,390	(-)	(-)	856,000	8.1	46.6	↑	(-)	Normal	46,XX [20]
	108	ET	66	M	(-)	596	12.7	91.9	30.3	33	14	11,290	(-)	(-)	1,225,000	8.9	8.9	↑	(+)	Normal	45,X,-Y [20]
	113	ET	47	M	(-)	619	14	90.2	30.8	34.1	12.9	9,700	(+)	(-)	1,552,000	7.7	47.8	↑	(+)	Normal	46,XY [20]
	128	ET	28	F	(-)	601	12.4	92.2	29.4	31.8	12.7	6,480	(-)	(+)	1,692,000	8.2	47.4	↑	(+)	Normal	46,XX [20]
Both	62	ET	68	F	(-)	672	10.1	87.5	28.5	32.6	13.9	7,070	(-)	(-)	1,195,000	8.1	42.7	↑	(+)	Alternative	46,XX [20]
Novel	75	ET	34	M	(-)	787	15.6	64.7	21	32.4	15.8	11,020	(-)	(-)	1,038,000	8.2	42.1	↑	(+)	Normal	46,XY [20]
Type 1	131	PMF	17	F	(-)	1,167	10.1	84.5	27	32.0	18.3	9,070	(-)	(+)	461,000	9.7	58.4	↑	(+)	Hyper	46,XX [9]

*Result by Sanger sequencing; †Cases with concurrent *CARL* and *JAK2* V617F mutations.
Abbreviations: Dx, diagnosis; JAK2, Janus kinase 2; LDH, lactose dehydrogenase; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, white blood cell; Seg, segmented neutrophils; Eosi, eosinophils; Baso, basophils; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; Mega, megakaryocytes; BM, bone marrow; Bx, biopsy; ET, essential thrombocythemia; PMF, primary myelofibrosis; Hyper, hypercellular; Normal, normal cellularity; Hypo, hypocellular; NT, not tested.

childbirth. BM findings did not change from those of the previous study. However, mutations were detected in the follow-up sample, even though neither *JAK2* V617F nor *CALR* mutation was detected in the first BM study. The patient started taking anagrelide (2 mg/day).

DISCUSSION

Detection of *CALR* mutations in *JAK2* V617F-negative ET and PMF patients has been helpful in diagnosing and predicting the prognosis of patients with previously difficult-to-characterize disease [4]. *CALR* mutations have been associated with longer survival times in PMF cases [9]. Several reports have also suggested that patients with *CALR* mutations have a lower incidence of thrombosis and hematologic progression than those with the *JAK2* V617F mutation [2, 6, 10].

In many studies, *CALR* mutational analysis was performed by PCR followed by Sanger sequencing [3, 4, 11, 12]. Some researchers have reported the use of other methods, such as targeted next generation sequencing (NGS) [13], fragment analysis [14], and high-resolution melting analysis (HRMA) [15, 16]. Sanger sequencing showed a sensitivity of 10% in *CALR* type 1 and type 2 mutations; it was less sensitive in samples with a low mutant allele burden [17]. The maximal reported sensitivity of HRMA in the detection of both *CALR* type 1 and type 2 mutations was 2.5% [17]. Jones et al. [13] who tested several methods for the detection of *CALR* mutations found that the most sensitive method was targeted NGS, which detected mutations down to a 1.25% mutant burden, followed by HRMA at 5%, fragment analysis at 5-10%, and Sanger sequencing at 10-25%. Methods for the detection of acquired mutations within hematologic malignancies showed a range of sensitivities. Here, we showed that our screening PCR can detect type 1 and type 2 mutations in the *CALR* gene, since the differential product sizes can be used to discriminate mutants from wild-type alleles.

The screening PCR detected *CALR* type 1 and type 2 mutants with a maximal sensitivity of 3.2% and <0.8%, respectively, compared with the higher sensitivities obtained by Sanger sequencing (6.3%, type 1 mutation). Because a specific primer for the detection of the type 2 mutation was used in the screening PCR, this system showed higher sensitivity in detecting the type 2 mutation than the type 1 mutation. In this study, the fragment analysis showed greater sensitivity in detecting the type 1 mutation (No. 32 and No. 74) than either Sanger sequencing or the screening PCR (Table 2). The fragment analysis identified two samples with low type 1 mutant burden (0.3% in No. 32 and 1.6% in No. 74); however, these mutations were not detected by using the other methods. Based on our results, maximal sensitivity in detecting the type 1 mutation was 6.3% by Sanger sequencing, 3.2% by the screening PCR, and <1% by the fragment analysis. The tumor burden of the two samples was too low to detect using Sanger sequencing and the screening PCR. This showed that the screening PCR could result in false negatives in cases with low allele burdens of *CALR* mutations. However, no false positive was detected in our screening PCR. Another limitation of the screening PCR is that it cannot detect a single nucleotide substitution or small deletion/insertion like the novel mutation found in one case in this study; however, the frequency of these mutations is rare. Therefore, this screening PCR would be useful as a screening test to detect the major *CALR* mutations in laboratories without sequencing equipment.

CALR mutations induce a frameshift through exon 9 deletions or insertions; type 1 and type 2 mutations constitute more than 80% of these mutations [18]. According to a previous report, at least 36 different indels account for the remaining variants [4, 13]. In this study, the frequency of *CALR* mutations, except for type 1 and type 2 mutations, was markedly low, probably owing to the small sample size. The majority of *CALR* mutations are believed to be heterozygous [4], and all mutations detected in the current study were also heterozygous. The prognostic value or clinical characteristics of zygosity have not yet been studied.

Other researchers have reported a slightly higher frequency of type 1 than type 2 mutation in the ET group (type 1: 45% vs. type 2: 39% [18] and type 1: 53.6% vs. type 2: 32.1% [12]). In this study, the frequency of the type 2 mutation (9/16, 56.3%) in ET patients was higher than that of type 1 (6/16, 37.5%). The small sample size of our study may have affected the discordant result with previous reports. *CALR* mutations in ET are generally associated with patients of a younger age, male sex, higher platelet count, lower hemoglobin levels, lower leukocyte counts, and lower incidence of thrombotic events. Tefferi *et al*. [9, 18, 19] reported that male sex was associated with only type 1 mutations, and that younger age and higher platelet counts were associated with type 2 mutations. Because of the small sample size, a statistical analysis of *CALR*-associated phenotypes was not performed for other disease groups.

Although *JAK2* and *CALR* mutations are considered mutually exclusive in patients with ET and PMF, co-expression of these mutations has been identified in a patient with PMF [20] and a patient with ET [21]. In the current study, two ET patients (No. 76 and No. 92) had co-existent *JAK2* V617F and type 1 *CALR* mutations. In another ET patient (No. 80), *JAK2* V617F and

type 2 *CALR* mutations were detected in follow-up BM samples. These findings provide additional exceptions to the rule of mutual exclusiveness of *JAK2* and *CALR* mutations. We suggest that an evaluation of *CALR* mutations be performed in ET patients with *JAK2* mutations and during follow-up studies. Interestingly, one ET patient (No. 62) had both type 1 and type 2 *CALR* mutations. This is the first such a case reported in an MPN patient.

In conclusion, the dual negative rate (*JAK2* V617F and *CALR*) was 14.8% in our ET group. In other words, approximately 85% of ET patients were diagnosed by these markers with demonstrated clonality. Our screening PCR is a rapid, sensitive, easy-to-use, and cost-effective method to screen major *CALR* mutations. We recommend using this rapid screening PCR for the identification of major *CALR* mutations in suspected MPN patients initially, and then, in the case of a negative result, using a more sensitive method for confirmation.

Authors' Disclosures of Potential Conflicts of Interest

No conflicts of interest relevant to this article were reported.

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