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Incidence, Clinical Features, and Prognostic Impact of *CALR* Exon 9 Mutations in Essential Thrombocythemia and Primary Myelofibrosis: An Experience of a Single Tertiary Hospital in Korea

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We evaluated the incidence, clinical characteristics, and prognostic impact of calreticulin (*CALR*) mutations in essential thrombocythemia (ET) and primary myelofibrosis (PMF) patients. In all, 48 ET and 14 PMF patients were enrolled, and the presence of *CALR* mutations was analyzed by direct sequencing. Patients were classified into three subgroups according to Janus kinase 2 (*JAK2*) V617F and *CALR* mutation status, and their clinical features and prognosis were compared. *CALR* mutations were detected in 15 (24.2%) patients, and the incidence increased to 50.0% in 30 *JAK2* V617F mutation-negative cases. These included 11 patients with three known mutations (c.1092_1143del [seven cases], c.1154_1155insTTGTC [three cases], and c.1102_1135del [one case]) and 4 patients with novel mutations. ET patients carrying *CALR* mutation were younger, had lower white blood cell counts, and experienced less thrombosis during follow-up than those carrying *JAK2* V617F mutation, while both patient groups showed similar clinical features and prognosis. In ET patients without *JAK2* V617F mutation, *CALR* mutation did not significantly affect clinical manifestation and prognosis. In conclusion, *CALR* mutation analysis could be a useful diagnostic tool for ET and PMF in 50% of the cases without *JAK2* V617F mutations. The prognostic impact of *CALR* mutations needs further investigation.

Key Words: CALR, Clinical feature, Essential thrombocythemia, Incidence, Mutation, Primary myelofibrosis, Prognosis

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The diagnosis of Philadelphia-negative myeloproliferative neoplasm (MPN) depends on bone marrow (BM) examination and Janus kinase 2 (*JAK2*) V617F mutation analysis [1-5]. *JAK2* exon 12 mutations and thrombopoietin receptor (*MPL*) mutations are useful markers to diagnose JAK2 V617F-negative MPN. However, since these mutations occur in <5% of *JAK2* V617F-negative MPNs, they have limited clinical relevance [6-8]. Recently, somatic mutations in exon 9 of calreticulin (*CALR*) were identified as a new diagnostic tool in *JAK2* V617F-negative MPNs, which were detected in 67% of the patients with essential thrombocythemia (ET) and 88% of patients with primary myelofibrosis (PMF) without *JAK2* V617F mutation [9]. Subsequent studies reported a trend of lower white blood cell (WBC) counts and hemoglobin levels, higher platelet counts, and more indolent clinical course in ET and PMF patients carrying *CALR* mutations than those carrying the *JAK2* V617F mutation [10-

15]. Furthermore, *CALR* mutations were proposed as a new diagnostic tool for ET and PMF in the revised WHO classification system [16].

However, favorable clinical outcome in MPN patients carrying *CALR* mutations has not been confirmed since myelofibrosis progression is also reported to have high incidence in ET patients with *CALR* mutations [15]. Moreover, studies in Asian populations are currently outnumbered since only 3 studies have evaluated this issue, but these studies have not included the Korean population [17-19]. Therefore, we analyzed the incidence, clinical features, and prognostic impact of *CALR* mutations in patients diagnosed as having ET and PMF at a single tertiary hospital in Korea.

Forty-eight patients diagnosed as having ET and 14 diagnosed as having PMF at Pusan National University Hospital from May 2007 to June 2014, according to the WHO diagnostic criteria classifications of 2008 [1] were included in this study. ET patients were managed with hydroxyurea only (32 patients), hydroxyurea and anagrelide (four patients), anagrelide only (six patients), or close monitoring without treatment (six patients). PMF patients were managed with hydroxyurea only (five patients), hydroxyurea and anagrelide (two patients), anagrelide only (two patients), oxymetholone only (two patients), or close monitoring without treatment (three patients). *JAK2* V617F mutation analysis was performed by using Seeplex JAK2 ACE Genotyping kit (Seegene, Seoul, Korea), and the median follow-up period in 62 patients was 7.73 months (range, 0.03-76.63 months). In 30 ET and PMF patients without *JAK2* V617F mutation, both *JAK2* exon 12 and *MPL* exon 9 mutation analyses were performed retrospectively by direct sequencing using in-house designed primers (JAK2-12-F, 5′-CTCCTCTTTGGAGCAATTCA-3′; JAK2-12-R, 5′-CCAATGTCACATGAATGTAAATCAA-3′; MPL-F, 5′-CCGAAGTCTGACCCTTTTTG-3′; MPL-R, 5′-ACAGAGCGAACCAAGAATGC-3′).

For *CALR* mutation analyses, PCR (F, 5′-GGCAAGGCCCTGAGGTGT-3′; R, 5′-CAGGCCTCAGTCCAGCCCTG-3′; 95°C for 1 min, 35 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec, followed by extension at 72°C for 7 min) was followed by direct sequencing. After confirming the presence of a single 267-base pair band (wild type) and multiple bands (mutation) in electrophoresis, direct sequencing was performed by using an ABI 3130 genetic analyzer (Applied Biosystems Inc., Foster city, CA, USA). Identified mutations were aligned with a reference sequence (NM_004343.3, c.1054_1254), while the novelty of the mutations was determined by screening a published database [9].

Clinical data of patients was obtained through retrospective review of electronic medical records, including gender, age, complete blood cell counts, BM cellularity, megakaryocyte counts, BM fibrosis grade, history of thrombosis, bleeding or acute myeloid leukemia transformation, survival rates at one year, overall survival (OS, the period between the initial diagnosis and last follow-up or death), and karyotype. Thus, prognostic scores according to Dynamic International Prognostic Scoring System (DIPSS) [20] were measured in 14 PMF patients. Both 48 ET and 14 PMF patients were classified into the following three subgroups according to *JAK2* V617F and *CALR* mutation status: (1), *JAK2*(-)/*CALR*(-); (2), *JAK2*(+)/*CALR*(-); (3), *JAK2*(-)/*CALR*(+). The clinical features, DIPSS scores (in PMF cases), and prognosis were compared among the three subgroups. This study was approved by the review board of the Pusan National University Hospital, and informed consent was obtained from all patients.

Among 62 patients, *JAK2* V617F mutation was detected in 32 (51.6%) patients, while *CALR* mutations were identified in 15 (24.2%) patients. Fifteen (24.2%) patients did not have *JAK2* or *CALR* mutations, while there were no patients with both mutations. Among 30 patients without the *JAK2* V617F mutation, *CALR* mutations were detected in 15 (50.0%) patients but *JAK2* exon 12 or *MPL* exon 9 mutations were not found in these patients (Table 1). Among 15 patients carrying *CALR* mutations, seven patients showed a 52-base pair deletion (c.1092_1143del), defined as the most frequent mutation (type 1) [9], three patients showed a 5-base pair insertion (c.1154_1155insTTGTC), defined as a frequently detected mutation (type 2) [9], while one patient showed a previously defined mutation type 4 (c.1102_1135del) [9]. Four patients were identified with novel mutations (c.1191_1199del, c.1116_1146del, c.1103_1148del, and c.1150_1151ins52) (Table 2).

Among 48 ET patients, those carrying *CALR* mutations were significantly younger (P=0.036) but had lower WBC counts

Table 1. Summary of *JAK2* V617F and *CALR* mutation profiles in a total of 62 ET and PMF patients

Patient subgroups	N of patients (%)		
	ET (N=48)	PMF (N=14)	Total (N=62)
JAK2(-)/*CALR*(-)	11 (22.9)	4 (28.6)	15 (24.2)
JAK2(-)/*CALR*(+)	12 (25.0)	3 (21.4)	15 (24.2)
JAK2(+)/*CALR*(-)	25 (52.1)	7 (50.0)	32 (51.6)
JAK2(+)/*CALR*(+)	0 (0.0)	0 (0.0)	0 (0.0)

Abbreviations: *JAK2*, Janus kinase 2; *CALR*, calreticulin; ET, essential thrombocythemia; PMF, primary myelofibrosis.

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Table 2. Demographic features and mutation profiles in 15 ET and PMF patients carrying *CALR* mutations

Case No.	Sex/Age	Diagnosis	Therapeutic regimen	*JAK2* mutation	*CALR* mutation results	*CALR* protein change	*CALR* mutation type	Chromosome
1	F/65	PMF	None	Negative	c.1191_1199del	p.Glu398_Asp400del	Novel	46,XX
2	F/59	ET	Anagrelide	Negative	c.1154_1155insTTGTC	p.Lys385fs*47	Type 2	46,XX
3	M/57	ET	Hydroxyurea	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XY
4	M/53	ET	Hydroxyurea	Negative	c.1116_1146del	p.Asp373fs*?	Novel	46,XY
5	F/62	ET	Hydroxyurea	Negative	c.1103_1148del	p.Lys368fs*?	Novel	46,XX
6	M/71	PMF	Anagrelide, hydroxyurea	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XY
7	F/63	ET	Hydroxyurea	Negative	c.1150_1151ins52	p.Asp384fs*1	Novel	46,XX
8	M/48	ET	Hydroxyurea	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XY
9	M/63	ET	Hydroxyurea	Negative	c.1154_1155insTTGTC	p.Lys385fs*47	Type 2	46,XY
10	M/39	ET	Anagrelide	Negative	c.1102_1135del	p.Lys368fs*?	Type 4	46,XY
11	M/57	ET	Hydroxyurea	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46, XY
12	M/56	PMF	Anagrelide	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XY,1qh+
13	F/47	ET	Hydroxyurea	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XX
14	M/22	ET	Hydroxyurea	Negative	c.1154_1155insTTGTC	p.Lys385fs*47	Type 2	46,XY
15	F/38	ET	Anagrelide	Negative	c.1092_1143del	p.Leu367fs*46	Type 1	46,XX

Classification of *CALR* mutation type and novelty was determined through searches in a recently published mutation database (Klampfl *et al*. [9], N Engl J Med 2013;369:2379-90).
Abbreviations: F, female; M, male; PMF, primary myelofibrosis; ET, essential thrombocythemia; *JAK2*, Janus kinase 2; *CALR*, calreticulin; fs, frameshift; del, deletion; ins, insertion.

Table 3. Demographic and clinical features of 48 essential thrombocythemia patients with respect to *JAK2* V617F and *CALR* mutation status

Variables	Mutation status		
	(1) *JAK2*(-)/*CALR*(-) (N=11)	(2) *JAK2*(+)/*CALR*(-) (N=25)	(3) *JAK2*(-)/*CALR*(+) (N=12)
Sex (M:F)*	6:05	12:13	7:05
Age, median (range)†	46.0 (19.0-85.0)	71.0 (33.0-81.0)§	55.0 (22.0-63.0)
WBC, $\times 10^9$/L, median (range)†	9.54 (6.47-18.33)	13.28 (2.56-100.00)§	8.47 (6.95-14.93)
Hb, g/dL, median (range)†	12.1 (7.9-17.0)	13.3 (7.2-18.1)	13.5 (9.6-15.9)
PLT, $\times 10^9$/L, median (range)†	698.0 (553.0-2251.0)	873.0 (557.0-1737.0)	965.0 (588.0-1530.0)
BM cellularity, median (range)†	60.0 (40.0 -100.0)	70.0 (60.0-100.0)	67.5 (40.0-90.0)
Megakaryocyte counts in BM biopsy, median (range)†	8.0 (5.0-13.0)§	12.0 (4.0-15.0)	10.5 (8.0-15.0)
BM fibrosis grade* (0/1/2/3/4)	3/8/0/0/0	2/16/6/1/0	1/10/1/0/0
Thrombosis developed at follow up*	2/11 (18.2%)	8/25 (32.0%)§	0/12 (0.0%)
AML transformation at follow up*	0/11 (0.0%)	0/25 (0.0%)	0/12 (0.0%)
Karyotype at diagnosis (normal/abnormal)*	11 (100.0%)/0 (0.0%)	23 (92.0%)/2 (8.0%)	12 (100.0%)/0 (0.0%)
Death at follow up*	1/11 (9.1%)	2/25 (8.0%)	0/12 (0.0%)
Estimated 1 yr survival rates‡	75.00%	84.20%	100.00%

P values were obtained from Chi-square test or Fisher's exact test*, Mann-Whitney U test† and Log rank test‡.
Statistical comparisons ([1] vs.[3] and [2] vs.[3]) were performed and those which showed statistical significances ($P<0.05$) were indicated with the superscript (§).
Abbreviations: JAK2, Janus kinase 2; CALR, calreticulin; WBC, white blood cells; PLT, platelet; BM, bone marrow.

($P=0.022$) and less thrombosis development during follow-up ($P=0.036$) than those with the *JAK2* V617F mutation. Patients carrying *CALR* mutations did not show significant differences in platelet counts, 1-yr survival rates, death rates, and OS ($P=0.341$) compared with those carrying the *JAK2* V617F mutation. Among the 23 ET patients without the *JAK2* V617F mutation, the

clinical features and OS (P=0.221) of the patients carrying *CALR* mutations were not significantly different from those without *CALR* mutations, although higher megakaryocyte counts in BM biopsy (P=0.020) were observed in the former group (Table 3).

In PMF, three patients carrying *CALR* mutations showed significantly lower WBC counts (median 4.30×10^9/L vs. 22.77×10^9/L, P=0.016) and tended to have lower hemoglobin levels (median 8.3 g/dL vs. 11.0 g/dL, P=0.087) than did seven patients carrying the *JAK2* V617F mutation. No significant differences in other clinical features, prognosis, and DIPSS scores (median 2.0 [0.0-3.0] in patients carrying *CALR* mutations vs. 1.0 [0.0-3.0] in those carrying the *JAK2* V617F mutation, P=0.400) were observed between the two subgroups. Among patients without the *JAK2* V617F mutation, no significant differences in clinical features, prognosis, and DIPSS scores (median 2.0 [0.0-3.0] vs. 2.5 [2.0-4.0], P=0.999) were observed between three patients with *CALR* mutations and four without *CALR* mutations, although significantly higher WBC counts were detected in the former group (median 4.30×10^9/L vs. 2.31×10^9/L, P=0.034).

Our data evidenced that the incidence of *CALR* mutations is 24.2% in ET and PMF, while the mutation frequency increased to 50.0% in patients without the *JAK2* V617F mutation, irrespective of the specific disease subtype. The incidence of *CALR* mutations reported herein is slightly lower than that reported by Klampfl *et al*. [9], while it is similar to the results in other studies (24% and 31% in ET and 20% in PMF) [10, 15]. Our results are also similar to previous reports on Asian populations (22.5% and 26.3% in ET and 21% in PMF) [17-19]; hence, the frequency of *CALR* mutation in ET and PMF is suggested to be independent of ethnic variations. Our data indicated that by introducing a *CALR* mutation analysis, 50% of the patients suspected of having ET and PMF but without the *JAK2* V617F mutation could be diagnosed correctly.

Consistent with previous reports, we demonstrated that ET patients carrying *CALR* mutations have distinct clinical features such as younger age, lower WBC counts, and lower thrombosis risk than those carrying the *JAK2* V617F mutation [9, 10, 14, 15]. In contrast with previous studies, we did not find significant differences in hemoglobin levels and platelet counts in ET patients with respect to their *JAK2* V617F and *CALR* mutation status [9-15]. Additionally, our data indicated that the *CALR* mutation status has no influence on clinical features, including prognosis in ET patients without the *JAK2* V617F mutation. Considering the small size of each subgroup, lack of statistical power might be a major limitation of our study, thus explaining the discrepancy with previous data. Similarly, the comparison of clinical features in PMF patients with respect to *JAK2* V617F and *CALR* mutation status might have been affected by the small size of each subgroup. Therefore, additional large-scale studies are required to confirm the clinical relevance of *CALR* mutations in ET and especially in PMF patients.

In conclusion, the incidence of *CALR* mutations in ET and PMF was 24.2% and increased to 50.0% in *JAK2* V617F-negative cases. ET patients carrying *CALR* mutations were younger, showed lower WBC counts, and experienced less thrombosis than did those carrying the *JAK2* V617F mutation, although no differences in other clinical features and prognosis were demonstrated. Furthermore, in ET patients without the *JAK2* V617F mutation, the presence of *CALR* mutations had no influence on clinical manifestation and prognosis. *CALR* mutation analysis can therefore be a useful additional diagnostic tool for ET and PMF, although the prognostic impact of *CALR* mutations should be addressed in more detail in future studies.

Authors' Disclosures of Potential Conflicts of Interest

No conflicts of interest relevant to this article were reported.

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