JAK2, *CALR*, and *MPL* mutation spectrum in Japanese patients with myeloproliferative neoplasms

Recurrent somatic mutations in the *JAK2*, *MPL*, and *CALR* genes have been described in patients diagnosed with Philadelphia-negative myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). These mutations are generally mutually exclusive, and their profiles in different disease entities are diverse. In PV, *JAK2* mutations exist in approximately 95% of patients. However, in ET and PMF patients, *JAK2*, *CALR*, and *MPL* mutations are present at frequencies of approximately 60%, 20%, and 5%, respectively.[1]

To further understand MPN pathogenesis associated with the ET and PMF induced by different gene alterations, classification and epidemiological examination of patients according to gene alterations have been performed. In ET, the *CALR* mutation is associated with a lower hemoglobin level, higher platelet count, lower leukocyte count, and younger age compared with the *JAK2*V617F mutation;[2-5] similar characteristics have been observed in PMF patients.[6,7] The *CALR* mutation is also associated with male predominance,[2,5] lower thrombosis risk,[4,6] and better overall survival;[6] however, these characteristics are not always evident and are diverse in some cases. The same gender ratio has been reported in a Chinese cohort of ET patients with *JAK2* and *CALR* mutations.[4] The variation between cohorts in the published data most likely reflects different genetic backgrounds in the different ethnic groups that were studied. In addition, because all analyses have been performed in Caucasian populations, with the exception of one study from China,[4] the epidemiological evidence regarding the Asian population is limited.

Here, we studied a Japanese MPN cohort that was previously characterized with respect to the *JAK2*V617F mutation. The cohort consisted of 66 PV, 112 ET, and 23 PMF patients, as defined by the 2008 World Health Organization (WHO) criteria.[8] Clinical and laboratory parameters were obtained at the time of first diagnosis or when genomic DNA samples were collected. This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Juntendo University School of Medicine (IRB#2012208 and #2013020).

All patient specimens that had previously been analyzed for the *JAK2* mutation[8] were assessed for *CALR* and *MPL* mutations using polymerase chain reaction (PCR)-based assays and subsequent deep-sequencing. In addition, the specimens that exhibited a *JAK2*V617F mutant allele frequency below 10% in the previous study were re-evaluated via deep sequencing (*Online Supplementary Appendix* and *Online Supplementary Table S1*). Re-evaluation identified one PV and 2 ET patients as negative for the *JAK2* mutation who a PCR-based assay had previously identified as *JAK2*V617F-positive with a low allele frequency. Conversely, *JAK2* mutations in MPN patients who were negative for *JAK2*, *MPL*, and *CALR* mutations by PCR-based assays were not identified by deep sequencing (see below for *MPL* and *CALR* mutation detection). Thus, *JAK2* mutations were found in 64 (97%) PV (including 3 exon 12

Table 1. Clinical and hematologic characteristics according to gene mutation status.

	ET (n=110**)				PMF (n=23)			
Mutation	*JAK2*	*CALR*	*MPL*	negative	*JAK2*	*CALR*	*MPL*	negative
Number of patients (n)	59	21	8	22	11	7	1	4
Male:female (n)	24:35	13:8	2:6	9:13	7:4	5:2	1:0	2:2
Age (years)	60.2 (19-83)	59.0 (33-86)	62.0 (32-81)	45.0 (26-81)	71.9 (52-88)	63.6 (55-81)	77	53.3 (38-76)
WBC ($\times 10^9$/L)	11.1 (5.2-52.3)	7.9 (4.1-12.7)	8.2 (4.9-11.3)	9.6 (4.8-21.9)	14.8 (2.2-52.8)	7.2 (3.2-12.3)	4.9	16.4 (5.4-44.1)
RBC ($\times 10^9$/L)	4783 (3420-7060)	4421 (2590-5570)	4364 (3600-4600)	4615 (3670-6160)	3876 (2230-5760)	3236 (2200-4111)	2960	3105 (1920-3680)
Hct (%)	42.0 (25.4-54.1)	41.3 (27.6-49.6)	39.6 (34.3-44.6)	40.9 (33.8-50.0)	32.8 (20.4-40.5)	29.2 (19.4-38.7)	24.8	28.1 (17.5-34.0)
Hb (g/dL)	13.7 (7.9-16.9)	13.6 (9.4-17.0)	12.7 (10.8-14.1)	13.4 (10.8-16.8)	10.3 (6.6-13.0)	9.1 (5.7-12.7)	7.8	9.4 (6.1-11.5)
MCV (fL)	88.6 (66.1-106.6)	93.7 (87.4-115.4)	90.8 (85.5-97.8)	89.3 (61.7-97.0)	86.2 (60.8-98.4)	90.2 (83.5-98.5)	83.8	90.7 (87.0-92.4)
Platelets ($\times 10^9$/L)	890 (486-1580)	1109 (496-2832)	1177 (800-1592)	910 (349-2337)	430 (70-1280)	477 (82-1491)	351	264 (2-855)
Thrombotic event*	9/55 (16.4%)	1/19 (5.3%)	1/8 (12.5%)	3/19 (15.8%)	2/6 (33.3%)	0/5 (0%)	0/1 (0%)	0/2 (0%)
Splenomegaly*	8/59 (13.6%)	0/20 (0%)	1/8 (12.5%)	1/20 (5.0%)	9/11 (81.8%)	4/6 (66.7%)	1/1 (100%)	3/4 (75%)

*WBC: white blood cell count; RBC: red blood cell count; Hct: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume. Values with a range are indicated by median values. *A subset of patients was evaluated. **The patients who exhibited mutations in two different genes were omitted.*

mutations), 61 (54.5%) ET, and 11 (47.8%) PMF patients in this cohort (Figure 1A).

The *MPL*W515K/L mutation was assessed using a newly developed allele-specific PCR technique called dual amplification refractory mutation system PCR (DARMS-PCR) and subsequent capillary electrophoresis.[9] All identified *MPL* mutations were further verified using deep sequencing. In addition, by screening MPN patients who were previously negative for *JAK2*, *MPL*, and *CALR* mutations we identified *MPL*W515K/L mutations below the detection limit of DARMS-PCR as well as other *MPL* mutations (*MPL*W515R).

Collectively, the *MPL*W515K/L/R mutation was identified in 9 (8.0%) ET and one (4.4%) PMF patients (Table 1 and Figure 1A), which was similar to the frequencies of 3%-8.3% that were found in Caucasian cohorts[2,3,5-7,10-13] but different from those in a Chinese cohort with a substantially lower frequency (1.2%).[4] We noted that one ET patient exhibited both the *MPL*W515K (allele frequency 50.7%) and W515L (allele frequency 2.5%) mutations.

The *CALR* mutation on exon 9 was examined using our in-house fragment analysis method (*Online Supplementary Appendix*). All identified *CALR* mutations were confirmed by deep sequencing. *CALR* mutations were identified in 22 (19.6%) ET and 7 (30.4%) PMF patients (Figure 1A), which was similar to the reported frequency in ET (15.5-28%)[2-6] and PMF (25%) patients.[7] In contrast, one study examined a Cypriot cohort and identified a frequency of 8.7%,[10] but patients with thrombocytosis were not classified according to the WHO 2008 criteria. Unlike the MPL mutation, the *CALR* mutation was present at similar frequencies in ET patients in both Japanese (19.6%) and Chinese (22.7%)[4] cohorts.

CALR mutations (n=29) were present in the following distribution: 11 type 1 (c.1092_1143del), 8 type 2 (c.1154_1155insTTGTC), one type 4 (c.1102_1135del), one type 22 (c.1120_1123del), one type 28 (c.1131_1152del), 2 type 33 (c.1154_1155insATGTC), and one type 34 (c.1154delinsCTTGTC) mutation, as well as four novel mutations (type 42-45; c.1100_1133del, c.1126_1144del, c.1153_1154insTCTGT, and c.1148_1154>GAC) (*Online Supplementary Table S2*). The *CALR* mutation in PMF patients is limited to types 1 and 2, and the type 1 mutation (n=6) is more frequent than the type 2 mutation (n=1), as observed in a Caucasian PMF cohort.[3] All novel *CALR* mutations generate a frame shift that converts the C-terminal amino acids from negatively to positively charged, as is the case for other mutations (*Online Supplementary Table S2*).

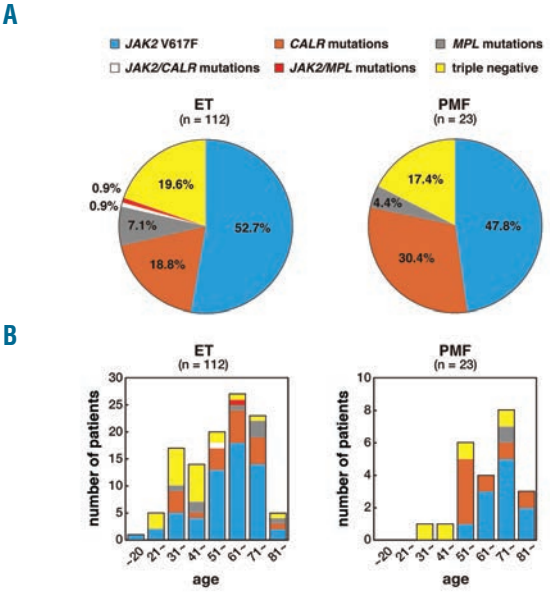

Figure 1. The *JAK2*, *MPL*, and *CALR* mutation frequencies in ET and PMF patients. (A) The *JAK2*, *MPL*, and *CALR* mutation frequencies in ET (n=112) and PMF (n=23) patients are shown. (B) The mutation statuses of different age groups are shown.

Although *JAK2*, *MPL*, and *CALR* mutations have been proposed to be mutually exclusive, we identified one ET patient with *JAK2*V617F and *MPL*W515L mutations and one ET patient with *JAK2*V617F and *CALR* mutations, which was consistent with recent reports that described a rare concomitant mutation of these gene mutations in Caucasian[7,14] and Chinese[4] cohorts. Finally, patient specimens that were negative for *JAK2*V617F, *MPL*W515K/L, and *CALR* exon 9 mutations by PCR-based assays were analyzed by deep sequencing of all the JAK2, MPL, and *CALR* exons (*Online Supplementary Appendix*). This analysis identified 22 (19.6%) ET and 4 (17.4%) PMF "triple-negative" patients (Figure 1A).

We compared the hematologic and clinical features of patients who were classified according to mutation status (Table 1), with the exception of 2 ET patients who harbored concurrent *JAK2* and MPL or *CALR* mutations (see above). In the ET patients, compared with the *JAK2*V617F mutation, the presence of the *CALR* or *MPL* mutation was associated with lower leukocyte and higher platelet counts (Table 1). The *CALR* mutation was also associated with a lower red blood cell count. These hematologic features are consistent with the features reported for different ethnic groups.[2-5] We observed a trend of male dominance among the ET patients with *CALR* mutations (male to female ratio 13:8) compared with the patients with *JAK2* mutations (male to female ratio 24:35), which is consistent with findings in Caucasian cohorts but inconsistent with findings in a Chinese cohort.[4]

We determined that the triple-negative ET patients (mean age 45.0 years old) were strikingly younger than the patients with other genotypes (Table 1 and Figure 1B). Adjusted P values for multiple comparisons of ages between triple-negative and other genotypes such as mutated *JAK2*, *CALR*, or *MPL* by Tukey-Kramer test were <0.001, 0.015, and 0.037, respectively. The mean age of the triple-negative ET patients exhibited a wide variation between the cohorts, ranging from 42 to 53 years.[3-5] The difference in age between the triple-negative patients and patients with other genotypes was more than 14 years, which was only observed in our cohort. In other cohorts, the difference in the ages of the triple-negative patients with the youngest age and patients with other genotypes with the second youngest age was, at most, five years.[15] Despite a lack of known clonal gene mutations, the triple-negative patients' bone marrow biopsies indicated apparent proliferation of megakaryocytes with a large and mature morphology, and their clinical characteristics corresponded to the WHO 2008 criteria for ET. Although it was a small cohort, we observed a very similar phenomenon in PMF patients; the difference in the ages of the triple-negative patients and patients with other genotypes was more than ten years (Table 1). Patients exhibiting myelofibrosis with no clonal mutations ("triple-negative") were diagnosed as PMF by excluding other diseases such as myelodysplasia through confirming no dysplasia in ery-

throid and/or myeloid lineages on bone marrow biopsy, chronic myeloid leukemia through defining Bcr-Abl negativity by FISH or PCR, and other diseases including autoimmune disorders through examining clinical records. This finding suggests that triple-negative ET and PMF patients in Japan have a distinct genetic background that facilitates the acceleration of disease onset compared with the Caucasian population.

In summary, we have shown that the mutation profile of our Japanese cohort of ET and PMF patients is comparable to that of Caucasian cohorts. However, the frequency of the *MPL* mutation differs between Asian populations, as demonstrated by the differences found between our cohort and a Chinese cohort. We identified four novel *CALR* mutations, all of which generate an altered C-terminus sequence that is commonly observed in patients with other *CALR* mutations, which implies that they are genuine mutations. The triple-negative ET and PMF patients were significantly younger than the patients with other genotypes in our cohort. The magnitude of this difference in Japan is much larger than that in other cohorts, which implies the presence of ethnic differences in ET and PMF development in triple-negative cases. Further genetic analysis would help clarify the genetic factors associated with ET development other than *JAK2*, *MPL*, and *CALR* mutations.

Shuichi Shirane,[1] Marito Araki,[2] Soji Morishita,[2] Yoko Edahiro,[1] Hiraku Takei,[1,3] Yongjin Yoo,[4] Murim Choi,[4] Yoshitaka Sunami,[1] Yumi Hironaka,[1] Masaaki Noguchi,[5] Michiaki Koike,[6] Naohiro Noda,[7] Akimichi Ohsaka,[2] and Norio Komatsu[1]

[1]Department of Hematology, Juntendo University School of Medicine, Tokyo, Japan; [2]Department of Transfusion Medicine and Stem Cell Regulation, Juntendo University Graduate School of Medicine, Tokyo, Japan; [3]Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan; [4]Department of Biomedical Sciences, Seoul National University College of Medicine, Korea; [5]Department of Hematology, Juntendo Urayasu Hospital, Chiba, Japan; [6]Department of Hematology, Juntendo Shizuoka Hospital, Japan; [7]Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Ibaraki, Japan

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Correspondence: komatsun@juntendo.ac.jp
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