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ORIGINAL ARTICLE

Genetic–pathologic characterization of myeloproliferative neoplasms

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Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by the proliferation of one or more myeloid lineages. The current study demonstrates that three driver mutations were detected in 82.6% of 407 MPNs with a mutation distribution of *JAK2* in 275 (67.6%), *CALR* in 55 (13.5%) and *MPL* in 6 (1.5%). The mutations were mutually exclusive in principle except in one patient with both *CALR* and *MPL* mutations. The driver mutation directed the pathologic features of MPNs, including lineage hyperplasia, laboratory findings and clinical presentation. *JAK2*-mutated MPN showed erythroid, granulocytic and/or megakaryocytic hyperplasia whereas *CALR*- and *MPL*-mutated MPNs displayed granulocytic and/or megakaryocytic hyperplasia. The lineage hyperplasia was closely associated with a higher mutant allele burden and peripheral cytosis. These findings corroborated that the lineage hyperplasia consisted of clonal proliferation of each hematopoietic lineage acquiring driver mutations. Our study has also demonstrated that bone marrow (BM) fibrosis was associated with disease progression. Patients with overt fibrosis (grade $\geqslant 2$) presented an increased mutant allele burden ($P<0.001$), an increase in chromosomal abnormalities ($P<0.001$) and a poor prognosis ($P<0.001$). Moreover, among patients with overt fibrosis, all patients with wild-type *JAK2*/*CALR*/*MPL* (triple-negative) showed genomic alterations by genome-wide microarray study and revealed the poorest overall survival, followed by *JAK2*-mutated MPNs. The genetic–pathologic characteristics provided the information for understanding disease pathogenesis and the progression of MPNs. The prognostic significance of the driver mutation and BM fibrosis suggests the necessity of a prospective therapeutic strategy to improve the clinical outcome.
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INTRODUCTION

BCR-ABL1-negative myeloproliferative neoplasms (MPNs) primarily include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1–3] The 2008 World Health Organization (WHO) classification system uses the presence of *JAK2* (V617F or exon 12 mutation) and *MPL* mutations as pathognomonic clues to the diagnosis of MPNs.[4] Recently, *CALR* mutations were reported in ET and PMF cases, which were mutually exclusive of *JAK2* or *MPL* mutations.[5,6] Therefore, it has been established that *CALR* as well as *JAK2* and *MPL* is the primary driver mutation in a *BCR-ABL1*-negative MPN.[7–10]

How a single driver mutation contributes to the pathogenesis of PV, ET and PMF, 'one-mutation-different diseases,' has been unclear.[11,12] The other dilemma is that each distinct MPN derived from a different genetic alteration has specific characteristics corresponding to the driver mutation. A pathologic standpoint such as lineage hyperplasia is advantageous to understanding disease on the basis of the driver mutation because the conceptual characteristics of

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MPNs are clonal proliferation. Bone marrow (BM) fibrosis is another pathologic characteristic because MPNs exhibit an intrinsic tendency to develop myelofibrosis; therefore, reticulin accumulation at diagnosis provides useful information regarding disease status.[13,14] Other surrogate markers of disease status are the increment of the mutant allele burden and the increase in cytogenetic aberrations.[15–17] In particular, PMF is the most heterogeneous, and copy number aberrations and gene mutations have been identified.[18,19]

In the current study, we identified *JAK2*, *MPL* and *CALR* mutations in a large cohort of Korean MPN patients and evaluated pathologic features such as lineage hyperplasia and fibrosis according to driver mutations. The mutant allele burden of each mutation, cytogenetic aberrations and clinical outcomes were analyzed to determine the genetic–pathologic characteristics of MPNs. The combination of classical and newly developed methods, including BM pathology, quantification of mutant allele and comparative genomic hybridization, and single-nucleotide polymorphism (CGH+SNP) arrays could improve understanding of the disease pathogenesis of MPNs.

MATERIALS AND METHODS

Patients and samples

All patients in this study provided written informed consent, and the study protocol was approved by the Institutional Review Board of The Catholic University of Korea. We studied 407 patients with MPN recruited from five tertiary hospitals in the Catholic Medical Center from 2009 to 2014. These hospitals included Seoul St Mary's Hospital and Yeouido St Mary's Hospital in Seoul, Uijeongbu St Mary's Hospital in Uijeongbu, Bucheon St Mary's Hospital in Bucheon, and Incheon St Mary's Hospital in Incheon, Korea. Clinical diagnosis of PV, ET and PMF was conducted in accordance with the 2008 WHO classifications.[4] Demographic, clinical and laboratory findings were gathered from medical records. A thrombosis event was defined as a major arterial event, such as acute myocardial infarction, stroke, transient ischemic attack and angina. Venous events included deep vein thrombosis, pulmonary thromboembolism, abdominal thromboembolism and Budd-Chiari syndrome.[20] Treatment was performed according to clinical diagnosis and associated symptoms. Low-risk PV patients were managed with low-dose aspirin plus phlebotomies to maintain the hematocrit level at $<45\%$. High-risk PV patients were treated with low-dose aspirin, phlebotomy and myelosuppressive therapy in the form of hydroxyurea. For low-risk ET patients, aspirin was administered unless platelet count $>1000\times10^9$/L or there was a contraindication for aspirin. High-risk patients were treated with low-dose aspirin and hyroxyurea or anagrelide. Symptomatic PMF patients were regularly monitored and managed with hydroxyurea, corticosteroid or transfusion as necessary. Allogeneic hematopoietic stem cell transplantation was performed in 11 PMF patients with intermediate-2 or high risk by dynamic international prognostic scoring system who in cases in which there were appropriate donors.

Molecular analysis of driver mutations in MPNs

Genomic DNA was obtained from BM aspirates or peripheral blood samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). An allele-specific, real-time PCR assay (Real-Q $JAK2^{V617F}$ Kit, BioSewoom, Seoul, Korea) was used to detect the *JAK2* V617F mutation using the amplification refractory mutation system principle.[21] All PCR reactions were run in duplicate. Single positive PCR reactions (C_T 35–40) were repeated in triplicate, and all replicates were required to be positive for the actual sample to be classified as mutation positive. The mutant allele burden was measured as the percentage of mutant allele with respect to total *JAK2* (mutant plus wild-type) using $JAK2^{V617F}$ Quantification Kit (BioSewoom). To identify the *JAK2* exon 12 mutation, a multiplex fragment analysis-based assay was used.[22] Sanger sequencing was conducted to confirm a region containing the entire exon 12 as previously described.[23] The mutant allele burden of the exon 12 mutation was measured by fragment analysis. The percentage of mutant DNA was calculated as the average percentage of the area of the mutant peak with respect to the total peak area (mutant plus wild-type peaks) after duplication.

To screen for common deletion and insertion mutations in the *CALR* gene, namely c.1092_1143del52 (type 1) and c.1154_1155insTTGTC (type 2), we conducted fragment analysis using PCR primers spanning exon 9 and a forward primer labeled with 6-FAM as previously described.[5] The percentage of mutant DNA was calculated as previously described. Sanger sequencing confirmed all identified mutations.

The $MPL^{W515L/K}$ mutation was analyzed using an allele-specific, real-time PCR assay (Real-Q $MPL^{W515L/K}$ Screening Kit, BioSewoom). Sanger sequencing was conducted to confirm the mutations.

Cytogenetic analysis

Chromosomal analysis. Chromosomal analyses were conducted on short-term cultures of BM specimens at diagnosis using standard conventional cytogenetic protocols. At least 20 metaphases were analyzed in each case, and clonal abnormalities were classified according to the 2013 International System for Human Cytogenetic Nomenclature as well as karyotype.[24]

Genome-wide microarray study and data analysis. We analyzed genomic alterations using the SurePrint G3 Human CGH+SNP Microarray 4×180 K kit according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). As a reference sample, Agilent's male or female genomic DNA (European normal individual) was used. The microarray slides were scanned at a three-micron resolution on an Agilent microarray scanner, and the raw data were extracted using Agilent Feature Extraction software V10.7.3.1. Raw data were analyzed using Agilent Genomic Workbench Software, CGH module 7.0.4.0 (Agilent Technologies). Genomic alterations were reported based on the following criteria: amplifications and deletions were scored when there was a 10-probe call with a minimum absolute average log2 ratio of 0.25, minimum genomic sizes of 0.5 Mb, and $<50\%$ overlap with known copy number alterations (Database of Genomic Variants). Mosaicism, coexisting minor populations with a major diploid population, was detected by visual inspection according to the following criteria: (i) a discontinuous line in the copy number state window compared with a continuous consistent line representing the major clonal population; (ii) intermediate values in the smooth signal, such as a minimum of 10 markers with a minimum absolute average log2 ratio of 0.1. A copy neutral-loss of heterozygosity (CN-LOH) larger than 5 Mb was considered using the LOH algorithm at the default threshold of 6.0.

Evaluation of pathologic features in patients' bone marrow

BM biopsies from the iliac crest were stained with hematoxylin and eosin for pathologic evaluation. Biopsies were reviewed by three pathologists with expertise in hematopathology. At time of re-evaluation, the pathologists were blinded to all clinical information

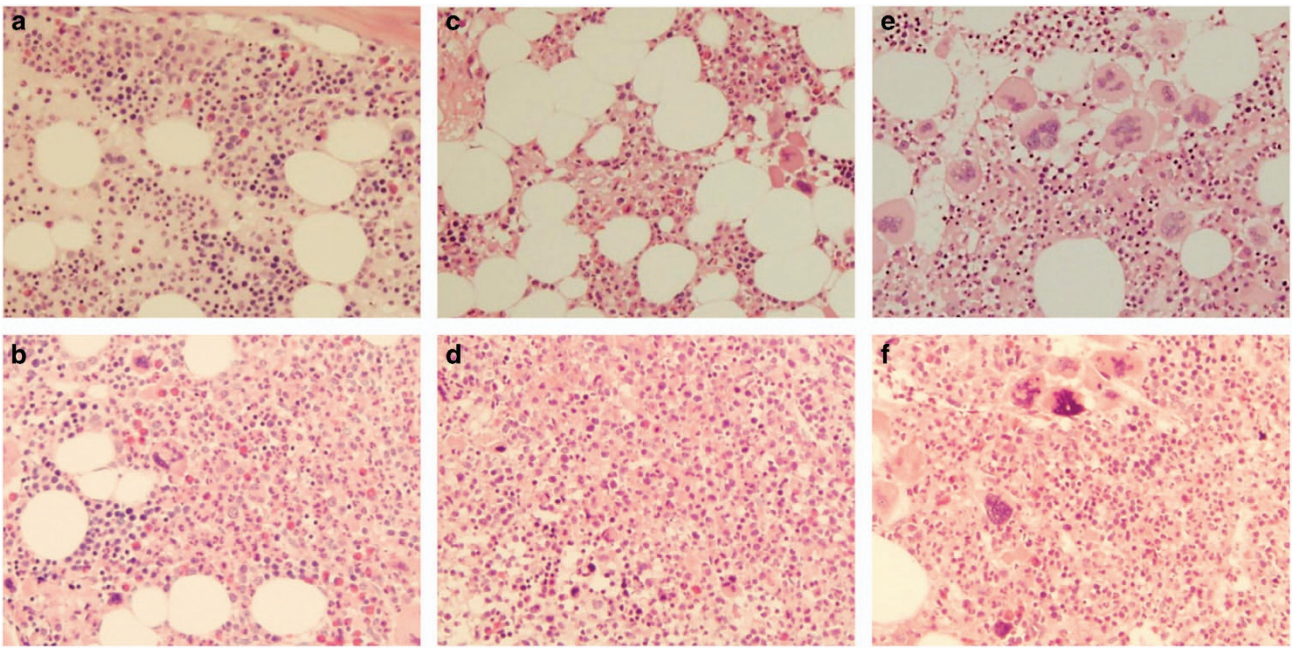

Figure 1 Representative bone marrow features of genetic–pathologic groups based on driver mutations and proliferation lineage as (**a**) *JAK2*-E, (**b**) *JAK2*-EGM, (**c**) *JAK2*-M and (**d**) *JAK2*-GM, (**e**) *CALR*-M and (**f**) *CALR*-GM (E, erythroid; G, granulocytic; M, megakaryocytic hyperplasia).

except for patient age. Each pathologist evaluated the following parameters of lineage hyperplasia: erythroid hyperplasia (E), granulocytic hyperplasia (G), megakaryocytic hyperplasia (M) and reticulin fibrosis (assessed on a 0–3 scale).[25] Despite its suggestive name, PMF does not generally show the evolvement of full-blown fibrosis. We were able to identify a preserved region in the BM of PMF patients to observe lineage hyperplasia. Finally, the lineage hyperplasia was grouped and described as follows: E(M), erythroid and/or megakaryocytic; M, megakaryocytic; GM, granulocytic and megakaryocytic; and EG(M), erythroid, granulocytic and/or megakaryocytic (Figure 1).

Statistical analysis

Differences between patients with *JAK2*, *CALR* and *MPL* mutations and patients without these three mutations (triple-negative) were analyzed by the Kruskal-Wallis test. The *P*-value was corrected by Bonferroni's method by multiple testing. The Mann-Whitney U-test was used for the analysis of non-parametric data, including comparison between two groups. The different histological characteristics and the consensus histological diagnosis were studied according to the distribution of mutations in the *JAK2*, *MPL*, and *CALR* genes using the chi-square test. Overall survival (OS) was estimated using the Kaplan-Meier product limit method, and the survival curves of different subgroups were compared by the log-rank test. Multivariate analysis of OS was conducted by Cox regression. All tests were two-tailed, and *P* was considered significant when <0.05. All statistical analyses were conducted using SPSS software (IBM SPSS Statistics 22, Armonk, NY, USA).

RESULTS

Driver mutations in MPNs

The total population of 407 patients with MPN included PV in 111 (27.3%), ET in 179 (44.0%), and PMF in 117 (28.7%). Somatic mutations were detected in 337 patients (82.8%) with a mutation distribution among *JAK2* in 275 (67.6%), *CALR* in 55 (13.5%), and *MPL* in 6 (1.5%). Wild-type *JAK2*/*CALR*/*MPL* (triple-negative) were detected in 70 (17.2%) patients. One ET patient displayed both *CALR* and *MPL* mutations (Supplementary Figure S1). The clinical and laboratory parameters among subgroups based on driver mutations are summarized in Table 1. On the basisof clinical diagnoses according to the WHO classification, somatic mutations were evident in 101 of 111 (91.0%) PV patients, 136 of 178 (76.4%) ET patients, and in 98 of 117 (83.8%) PMF patients. In PV patients, only *JAK2* mutations were observed, which were subdivided into *JAK2* V617F mutations ($n=95$, 94.1%) and exon 12 mutations ($n=6$, 5.9%). In ET patients, *JAK2* V617F, *CALR*, and *MPL* mutations were observed in 53.4%, 21.9% and 1.7% of patients, respectively. In PMF patients, *JAK2* V617F, *CALR* and *MPL* mutations were observed in 67.5%, 13.7% and 2.6% of patients, respectively.

Age of onset was latest in *JAK2*-mutated patients (median 66 years) when compared with *CALR*-mutated and triple-negative patients ($P=0.001$ and $P<0.001$, respectively). The median leukocyte count was highest in *JAK2*-mutated MPN (11.91×10^9/L) compared with *CALR*-mutated (8.58×10^9/L, $P<0.001$), *MPL*-mutated (5.89×10^9/L, $P=0.002$) and triple-negative patients (7.75×10^9/L, $P<0.001$). The hemoglobin levels were highest in *JAK2*-mutated patients, followed by *CALR*-mutated, triple-negative and *MPL*-mutated patients (14.7, 12.6, 11.1 and 7.8 g/dL, respectively). Hematocrit levels were highest in *JAK2*-mutated patients, followed by *CALR*-mutated, triple-negative, and *MPL*-mutated patients (44.4%, 38.4%, 33.6% and 25.3%,

Table 1 Demographic, laboratory and clinical characteristics of 407 patients with *BCR/ABL1*-negative MPNs according to driver mutations

	Total	*JAK2 mutated*	*CALR mutated*	*MPL mutated*	*Triple negative*
Number of patients (%)	407 (100)	275 (67.6)	55 (13.5)[a]	6 (1.5)[a]	70 (17.2)
Males (%)[b]	192 (47.2)	124 (45.1)	23 (41.8)	3 (50.0)	42 (60.0)
Age, years[c]	63 (20–89)	66 (22–89)	57.5 (20–89)	69 (53–81)	53 (25–83)
Leukocytes, $\times 10^9$/L[d]	10.37 (0.75–177.54)	11.91 (2.20–177.54)	8.58 (4.84–30.61)	5.89 (2.23–10.99)	7.75 (0.75–89.40)
Hemoglobin, g/dL[e]	13.5 (4.1–23.3)	14.7 (6.2–22.6)	12.6 (7.5–16.1)	7.8 (6.0–13.1)	11.1 (4.1–23.3)
Hematocrit[f]	40.7 (12.4–69.1)	44.4 (19.7–69.1)	38.4 (22.9–47.0)	25.3 (18.8–39.8)	33.6 (12.4–66.3)
Red blood cells, $\times 10^{12}$/L[g]	4.63 (1.36–9.72)	5.12 (1.89–9.72)	4.16 (2.25–5.32)	2.68 (2.13–4.27)	3.75 (1.36–6.81)
Platelets, $\times 10^9$/L[h]	676.5 (13–3268)	643 (13–3268)	898 (49–1795)	510.5 (150–1256)	668.5 (13–1630)
Thrombosis events (%)[b]	26 (6.4)	23 (8.4)	0 (0.0)	1 (16.7)	2 (2.9)
Clinical diagnosis (%)[b]					
PV	111 (27.3)	101 (36.7)	0 (0.0)	0 (0.0)	10 (14.3)
ET	179 (44.0)	95 (34.5)	39 (70.9)	3 (50.0)	41 (58.6)
PMF	117 (28.7)	79 (28.7)	16 (29.0)	3 (50.0)	19 (27.0)
Proliferation lineage (%)[b]					
E(M)	45 (11.1)	37 (13.5)	0 (0.0)	0 (0.0)	8 (11.4)
EG(M)	88 (21.6)	86 (31.3)	0 (0.0)	0 (0.0)	2 (2.9)
M	164 (40.3)	80 (29.1)	36 (65.5)	5 (83.3)	42 (60.0)
GM	110 (27.0)	72 (26.2)	19 (34.5)	1 (16.7)	18 (25.7)

Abbreviations: EG(M), erythroid, granulocytic and/or megakaryocytic hyperplasia; E(M), erythroid and/or megakaryocytic hyperplasia; ET, essential thrombocythemia; GM, granulocytic and megakaryocytic hyperplasia; M, megakaryocytic hyperplasia; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; PV, polycythemia vera; triple negative, *JAK2/CALR/MPL* wild type.
[a]An ET patient with both *MPL* and *CALR* mutations was excluded in this column.
[b]Percentage of each mutant group.
[c]*JAK2* compared with *CALR* ($P=0.001$), *JAK2* compared with triple negative ($P<0.001$).
[d]*JAK2* compared with *CALR* ($P<0.001$), *JAK2* compared with *MPL* ($P=0.002$), *JAK2* compared with triple negative ($P<0.001$).
[e]*JAK2* compared with *CALR* ($P<0.001$), *JAK2* compared with *MPL* ($P=0.001$), *JAK2* compared with triple negative ($P<0.001$), *CALR* compared with *MPL* ($P=0.006$).
[f]*JAK2* compared with *CALR* ($P<0.001$), *JAK2* compared with *MPL* ($P=0.001$), *JAK2* compared with triple negative ($P<0.001$), *CALR* compared with *MPL* ($P=0.003$), *CALR* compared with triple negative ($P=0.046$).
[g]*JAK2* compared with *CALR* ($P<0.001$), *JAK2* compared with *MPL* ($P=0.001$), *JAK2* compared with triple negative ($P<0.001$), *CALR* compared with *MPL* ($P=0.005$).
[h]*JAK2* compared with *CALR* ($P<0.001$), *CALR* compared with triple negative ($P<0.001$).

respectively). Red blood cell counts were highest in *JAK2*-mutated patients followed by *CALR*-mutated, triple-negative, and *MPL*-mutated patients (5.12×10^{12}/L, 4.16×10^{12}/L, 3.75×10^{12}/L and 2.68×10^{12}/L, respectively). Platelet counts were highest in *CALR*-mutated MPN patients followed by triple-negative, *JAK2*-mutated and *MPL*-mutated groups (898×10^9, 668.5×10^9, 643×10^9 and 510.5×10^9/L, respectively).

Driver mutations and lineage hyperplasia

The hyperplastic lineage of BM was dependent on driver mutations. *JAK2*-mutated and triple-negative MPN included all types of lineage hyperplasia, E(M), EG(M), M and GM, whereas *CALR*- and *MPL*-mutated MPNs displayed only two (M and GM). A clinical diagnosis of PV primarily involved *JAK2*-mutated and triple-negative E(M) and EG(M). ET and PMF were included in the EG(M), M and GM and revealed all types of genetic mutations (Supplementary Table S1).

JAK2-mutated MPN. *JAK2* mutations were identified in 275 patients with 97.5% of V617F ($n=268$) and 2.5% of exon 12 ($n=7$) mutations (Table 2). The V617F mutation showed all four types of lineage hyperplasia, E(M), EG(M), M and GM, whereas the exon 12 mutation showed only E(M) and EG(M). The laboratory parameters closely reflected the hyperplastic lineage (Figure 2). The median leukocyte count was higher in patients with granulocytic hyperplasia (EG(M) 16.16×10^9/L, GM 15.67×10^9/L) than in patients without granulocytic hyperplasia (E(M) 9.87×10^9/L, M 8.63×10^9/L; $P<0.001$). Red blood cell counts were higher in patients with erythroid hyperplasia (E(M) 6.52×10^{12}/L, EG(M) 6.54×10^{12}/L) than in patients without erythroid hyperplasia (M 4.35×10^{12}/L, GM 4.29×10^{12}/L; $P<0.001$). Platelet counts were higher in patients with GM and M (755×10^9/L, 743.5×10^9/L), followed by EG(M) and E(M) (499.5×10^9/L, 391.5×10^9/L; $P=0.012$).

The mutant allele burden of V617F was widely distributed (1.8–98.6%) although localized below 50% in the exon 12 mutation (34.5–49.4%). Among the *JAK2*-mutated MPNs, V617F revealed a higher mutant allele burden than exon 12 although the difference was not statistically significant. When the comparison was restricted in E(M) and EG(M), the mutant allele burden was significantly higher in V617F than in exon 12

Table 2 Comparison between patients with *JAK2* V617F and exon 12 mutations

	JAK2 mutated	*JAK2 V617F*	*JAK2 exon 12*	*P-value*
Number of patients (%)[a]	275 (100)	268 (97.5)	7 (2.5)	
Age, years	66 (22–89)	66 (22–89)	66 (46–76)	0.927
Males (%)	124 (45.1)	122 (45.5)	2 (28.6)	0.374
Leukocytes, $\times 10^9$/L	11.91 (2.20–177.54)	12.00 (2.20–177.54)	8.20 (6.17–22.32)	0.095
Hemoglobin, g/dL	14.7 (6.2–22.6)	14.7 (6.2–22.6)	18.3 (13.7–21.1)	0.012
Hematocrit	44.4 (19.7–69.1)	43.9 (19.7–69.1)	49.9 (46.2–59.3)	0.037
Red blood cells, $\times 10^{12}$/L	5.12 (1.89–9.72)	5.06 (1.89–9.72)	6.90 (5.83–8.50)	0.004
Platelets, $\times 10^9$/L	643 (13–3268)	650 (13–3268)	281 (58–310)	0.001
Proliferation lineage (%)[a]				<0.001
E(M)	37 (13.5)	32 (11.6)	5 (1.8)	
EG(M)	86 (31.3)	84 (30.5)	2 (0.7)	
M	80 (29.1)	80 (29.9)	0 (0.0)	
GM	72 (26.2)	72 (26.2)	0 (0.0)	
Mutant allele burden, %				
Total	64.2 (1.8–98.6)	66.8 (1.8–98.6)	43.5 (34.5–49.4)	0.052
E(M)+EG(M)	84.7 (13.3–98.6)	85.3 (13.3–98.6)	43.5 (34.5–49.4)	<0.001
Clinical diagnosis (%)[a]				0.021
PV	101 (36.7)	95 (34.5)	6 (2.2)	
ET	95 (34.5)	95 (34.5)	0 (0.0)	
PMF	79 (28.7)	78 (28.4)	1 (0.4)	

Abbreviations: EG(M), erythroid, granulocytic and/or megakaryocytic hyperplasia; E(M), erythroid and/or megakaryocytic hyperplasia; ET, essential thrombocythemia; GM, granulocytic and megakaryocytic hyperplasia; M, megakaryocytic hyperplasia; PMF, primary myelofibrosis; PV, polycythemia vera.
[a]Percentage of total *JAK2*-mutated patients.

(84.9% compared with 43.45%, $P=0.001$). These findings were expected because homozygosity for the V617F mutation is displayed by ~30% of PV or PMF patients[26] although homozygosity is rare in exon 12 mutations.[23,27] The *JAK2* V617F mutant allele burden was lowest in the M (37.3%), followed by GM, E(M) and EG(M) (68.9%, 76.0% and 89.2%, respectively, $P<0.001$).

CALR-mutated MPN. *CALR* mutations were identified in 55 patients with two recurrent variants: *L367fs*46*, which resulted from a 52-bp deletion flanked by seven base pairs of identical sequences (type 1); and *K385fs*47*, which resulted from a 5-bp insertion that represented an inverse duplication of the five nucleotides preceding the insertion (type 2). In these 55 patients, type 1 and type 2 mutations were identified in 35 (63.6%) and 16 (29.1%) patients, respectively. Four patients (7.3%) revealed unique indel mutations (Supplementary Table S2). Platelet counts were higher in type 2 than in type 1 (1103×10^9/L compared with 836×10^9/L, $P=0.033$). The *CALR* mutant allele burden ranged widely, from 17.75 to 93.24%. The type 1 mutation revealed a higher mutant allele burden than type 2 (52.63% compared with 38.20%, $P<0.001$; Table 3). Similar to the *JAK2*-mutated MPNs, laboratory parameters in *CALR*-mutated patients were strongly affected by their lineage hyperplasia. Median leukocyte counts were higher in GM (11.50×10^9/L) than M (7.43×10^9/L, $P<0.001$). Red blood cell counts did not differ between the two groups. The *CALR* mutant allele burden was higher in GM (57.23%) than M (45.87%, $P=0.002$; Figure 2).

BM fibrosis

The grade of BM fibrosis was divided into minimal fibrosis (<grade 2) and overt fibrosis (⩾grade 2). There was no difference in the occurrence rate of overt fibrosis between *JAK2*- and *CALR*-mutated and triple-negative patients (22.2%, 27.1% and 29.3%, respectively). *JAK2*-GM and *CALR*-GM showed a high rate of overt fibrosis (46.0 and 42.1%), followed by *JAK2*-M (17.5%), *CALR*-M (17.2%) and *JAK2*-EG(M) (10.4%; $P<0.001$). Notably, none of the *JAK2*-E(M) patients presented overt fibrosis (Table 4). The red blood cell count was significantly lower in patients with overt fibrosis, and the mutant allele burden was significantly higher in patients with overt fibrosis than in patients without overt fibrosis (Supplementary Figure S2).

Cytogenetic aberrations

Chromosomal analyses were performed for 279 samples. Of these, 50 (17.9%) revealed clonal abnormalities. Loss of 20q ($n=9$, 18.0%) was most frequently detected, followed by trisomy 8 ($n=8$, 16.0%), loss of Y chromosome ($n=8$, 16.0%) and loss of 13q ($n=4$, 8.0%). Frequencies of chromosomal abnormalities were highest in PMF (31.1%), followed by PV (12.1%) and ET (11.4%; $P<0.001$). The incidence of chromosomal abnormalities did not differ among *JAK2*- and *CALR*-mutated and triple-negative MPNs (19.5, 11.9 and 18.8%, $P>0.05$). However, the chromosomal abnormalities were more commonly developed in patients with overt fibrosis than in patients without overt fibrosis (37.5% compared with 12.0%, $P<0.001$). In *JAK2*-mutated MPNs, the chromosomal

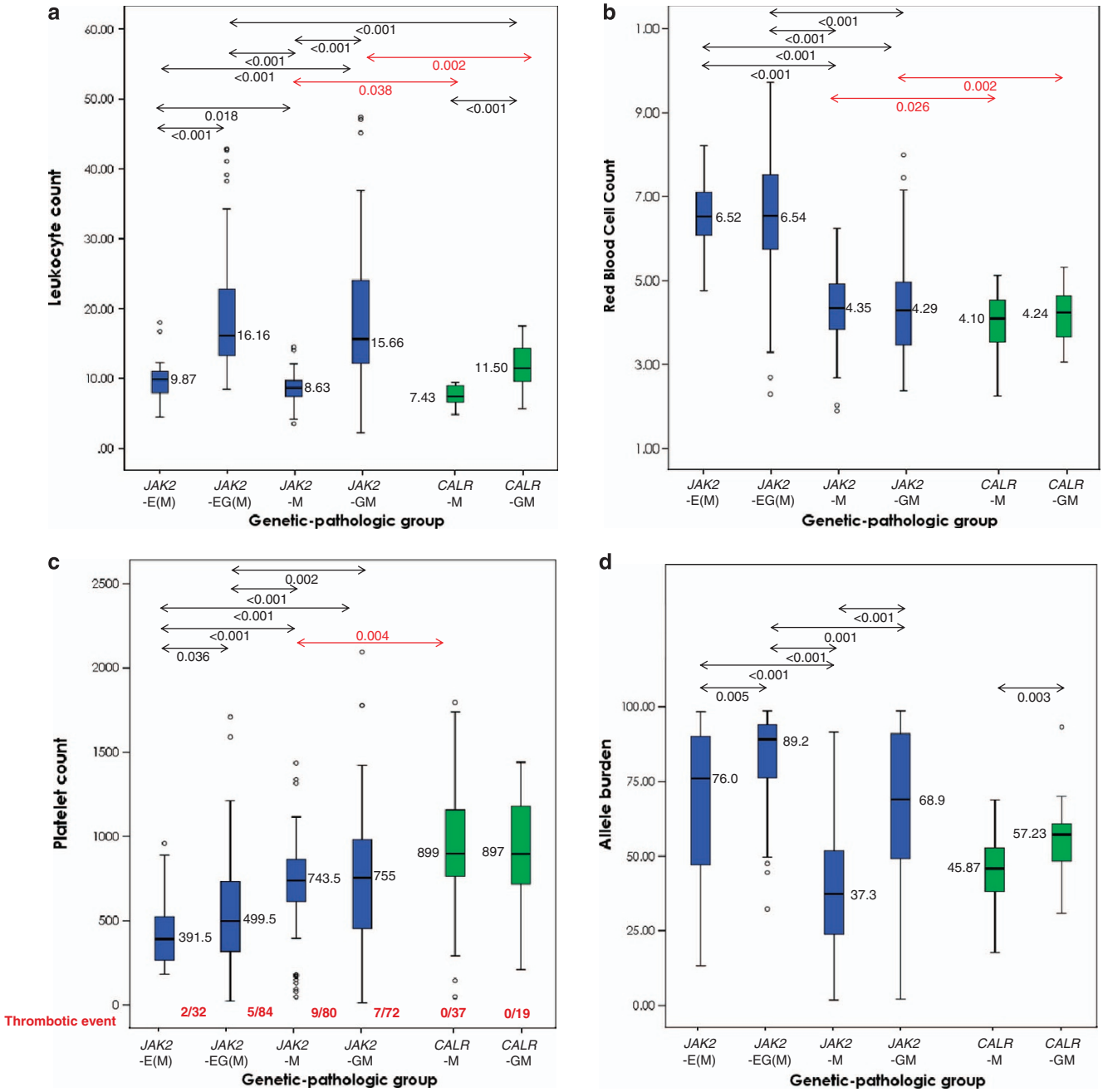

Figure 2 Comparison of peripheral cytosis and allele burden among genetic–pathologic groups. The genetic–pathologic group is closely associated with the peripheral blood cell count (**a**–**c**). The incidence of major thrombotic events in genetic–pathologic groups is indicated in **c**. Mutant allele burden represents clonal proliferation of hematopoietic lineage with each driver mutation (**d**).

abnormalities were more frequently observed in patients with overt fibrosis (45.9% compared with 12.7%, $P<0.001$). Similarly, the chromosomal abnormalities were more frequently observed in *CALR*- mutated patients combined with overt fibrosis than in patients without that combination (36.4% compared with 3.2%, $P=0.013$). In triple-negative MPNs, the incidence of chromosomal abnormalities did not differ between overt and minimal fibrosis (20.0% compared with 18.2%, $P>0.05$). Among 13 triple-negative patients with overt fibrosis, only two cases showed an abnormal karyotype with sole del(13q) and trisomy 21. Chromosomal analysis was normal in nine cases and failed to yield metaphase cells in two cases. Therefore, we searched for genomic alterations using CGH+SNP array in those patients. Genomic alterations were detected in all cases investigated. A total of 60 alterations were identified, including 44 CN-LOHs, 12 chromosomal deletions and 4 chromosomal amplifications (Figure 3). One case had only one aberration, and the other cases had more than three (median 5, range 1–7). The size of each aberration ranged from approximately 1.4 to 159.0 Mb (median 4.2 Mb).

Table 3 Clinical and laboratory parameters in patients with *CALR* mutation, stratified by the presence of recurrent somatic mutations

	CALR mutated	*CALR type 1*	*CALR type 2*	*CALR, other*	*P-value*[a]
Number of patients (%)[b]	55 (100)	35 (63.6)	16 (29.1)	4 (7.3)	
Age, years	57.5 (20–89)	60 (20–89)	56 (30–72)	71 (41–79)	0.234
Males (%)	23 (41.8)	12 (34.3)	8 (50.0)	3 (75.0)	0.374
Leukocytes, $\times 10^9$/L	8.58 (4.84–30.61)	9.12 (4.84–30.61)	7.24 (5.66–16.57)	10.07 (7.43–15.36)	0.208
Hemoglobin, g/dL	12.6 (7.5–16.1)	12.4 (7.5–16.1)	13.1 (8.9–15.3)	13.4 (8.9–14.4)	0.049
Hematocrit (%)	38.4 (22.9–47.0)	37.2 (22.9–46.6)	40.25 (27.2-47.0)	40.35 (29.1–42.7)	0.040
Red blood cells, $\times 10^{12}$/L	4.16 (2.25–5.32)	4.07 (2.25–5.32)	4.34 (2.87–5.25)	4.12 (2.72–4.59)	0.133
Platelets, $\times 10^9$/L	895 (49–1795)	836 (146–1739)	1,103 (49–1795)	1,145.5 (819–1441)	0.033
Proliferation lineage (%)[b]					0.107
M	37 (66.1)	21 (37.5)	14 (25.0)	2 (3.6)	
GM	19 (33.9)	14 (25.0)	3 (5.4)	2 (3.6)	
Mutant allele burden, %	48.23 (17.75–93.24)	52.63 (21.71–70.10)	38.2 (17.75–93.24)	52.08 (43.50–68.76)	<0.001
Clinical diagnosis (%)[b]					0.214
ET	40 (71.4)	23 (41.1)	14 (25.0)	3 (5.4)	
PMF	16 (28.6)	12 (21.4)	3 (5.4)	1 (1.8)	

Abbreviations: ET, essential thrombocythemia; GM, granulocytic and megakaryocytic hyperplasia; M, megakaryocytic hyperplasia; PMF, primary myelofibrosis.
[a]*P*-values from comparison of type 1 and type 2.
[b]Percentage of total *CALR*-mutated patients.

Loss of 5q (deletion and CN-LOH) was isolated in 6 cases, and loss of 13q was observed in five cases. CN-LOHs of chromosomes 2, 3, 4, 11, 18 and X were detected in more than three patients. Loss of known potential leukemogenic genes, including *TET2* (4q24), *CUX1* (7q22.1), *ETV6*, *CDKN1B* (12p13), *RB1* (13q14.2) and *SOCS2* (12q22), were identified in six patients.

Clinical outcomes

Major thrombotic events were recorded in 26 (6.4%) patients. These patients had higher platelet counts than the 381 patients who did not experience major thrombotic events (807.5×10^9/L compared with 668×10^9/L, $P = 0.008$). Thrombosis events primarily occurred in *JAK2*-mutated ($n = 23$) patients. The other patients with thrombosis events were either *MPL* mutated ($n = 1$) or triple negative ($n = 2$; $P = 0.043$). Thrombosis events were not recorded in *CALR*-mutated patients although the median platelet count was highest in that population. Among the V617F-mutated patients, 8.6% (23/269) experienced thrombosis events; their platelet counts were higher than in patients without the mutation (614×10^9/L compared with 835×10^9/L, $P = 0.001$). Thrombosis was not recorded in exon 12-mutated patients.

The estimated mean survival of the study population was 68 months with a 95% confidence interval of 65–70 months. PMF patients had a poorer OS than PV and ET patients ($P < 0.001$; Figure 4a), and triple-negative patients had a poorer OS than *JAK2*- and *CALR*-mutated MPNs ($P = 0.003$; Figure 4b). In *JAK2*-mutated patients, lineage hyperplasia affected the OS that *JAK2*-GM revealed as the poorest OS ($P = 0.006$; Figure 4c). Conversely, the OS of *CALR*-mutated patients did not differ by lineage hyperplasia. Patients with overt fibrosis revealed poorer OS than patients with minimal fibrosis ($P < 0.001$; Figure 4d). In *JAK2*-mutated and triple-negative patients, overt fibrosis affected the OS ($P < 0.001$ and $P < 0.001$) whereas overt fibrosis did not affect OS in *CALR*-mutated patients (Figure 4e). Patients with abnormal chromosomes had a poorer OS ($P = 0.001$; Figure 4f). Among patients with overt fibrosis, triple negative had the poorest OS compared with *JAK2*- and *CALR*-mutated patients ($P < 0.001$). In multivariate analyses, we included age, sex, the WHO diagnosis, lineage hyperplasia, abnormal chromosomes, driver mutation and overt fibrosis as covariates; driver mutation and overt fibrosis predicted poor OS (Supplementary Table S3). PMF patients who received hematopoietic stem cell transplantation ($n = 11$) survived the follow-up period.

DISCUSSION

The current study presents the molecular distribution among actual incidences of *JAK2*, *CALR*, and *MPL* somatic mutations in 407 MPN patients recruited from five hospitals in the Catholic Medical Center. Results from previous studies showed a similar distribution of acquired mutation as driver causes of three distinct clonal MPN disease entities.[5,6] *JAK2* mutations were observed in 67.6% of 407 patients with clinical presentation of PV (36.7%), ET (34.5%), and PMF (28.7%). *CALR* and *MPL* mutations occurred in 13.5% and 1.5% of patients and were not detected in PV patients. The coexistence of *JAK2*, *CALR* and/or *MPL* mutations was extremely rare. Only two rare cases were reported, a PMF with *JAK2* V617F and *CALR* type 1 mutations[28] and an ET with *MPL* W515R and *CALR* type 1 mutations.[29] We first identified one ET patient with both *MPL* W515L and *CALR* type 2 mutations. The mutant allele burden of the *CALR* gene was higher than the burden of the *MPL* gene in this case, which suggests that the *MPL*

Table 4 Mutant allele burden and chromosomal abnormality according to bone marrow fibrosis in each genetic–pathologic group

	Total	*JAK2-E(M)*	*JAK2-EG(M)*	*JAK2-M*	*JAK2-GM*	*CALR-M*	*CALR-GM*	P-*value*[a]
Number of patients	256	21	67	57	63	29	19	<0.001
Overt fibrosis[b] (%)	59 (23.0)	0 (0.0)	7 (10.4)	10 (17.5)	29 (46.0)	5 (17.2)	8 (42.1)	
Minimal fibrosis[c] (%)	197 (77.0)	21 (100.0)	60 (89.6)	47 (82.5)	34 (54.0)	24 (82.8)	11 (57.9)	
Chromosomal abnormality, %[d]								<0.001
In overt fibrosis	43.8	0.0	40.0	62.5	41.7	40.0	33.3	
In minimal fibrosis	11.0	0.0	14.5	12.5	16.1	0.0	10.0	
Mutant allele burden, %								<0.001
In overt fibrosis	72.15 (6.30–98.00)	NA	90.05 (74.00–98.00)	66.15 (6.30–91.50)	84.70 (21.20–97.20)	52.63 (38.20–56.38)	62.51 (48.15–93.24)	
In minimal fibrosis	52.90 (2.15–98.60)	74.40 (13.30–98.30)	89.30 (44.50–98.20)	33.05 (2.90–70.90)	57.90 (2.15–98.60)	41.81 (17.75–68.76)	49.55 (30.87–60.44)	

Abbreviations: EG(M), erythroid, granulocytic and/or megakaryocytic hyperplasia; E(M), erythroid and/or megakaryocytic hyperplasia; GM, granulocytic and megakaryocytic hyperplasia; M, megakaryocytic hyperplasia; NA, not applicable.
[a]*P* values from comparison of overt fibrosis and minimal fibrosis.
[b]Fibrosis grade <2.
[c]Fibrosis grade $\geqslant 2$.
[d]Percentage of patients with chromosomal abnormalities in each fibrosis group.

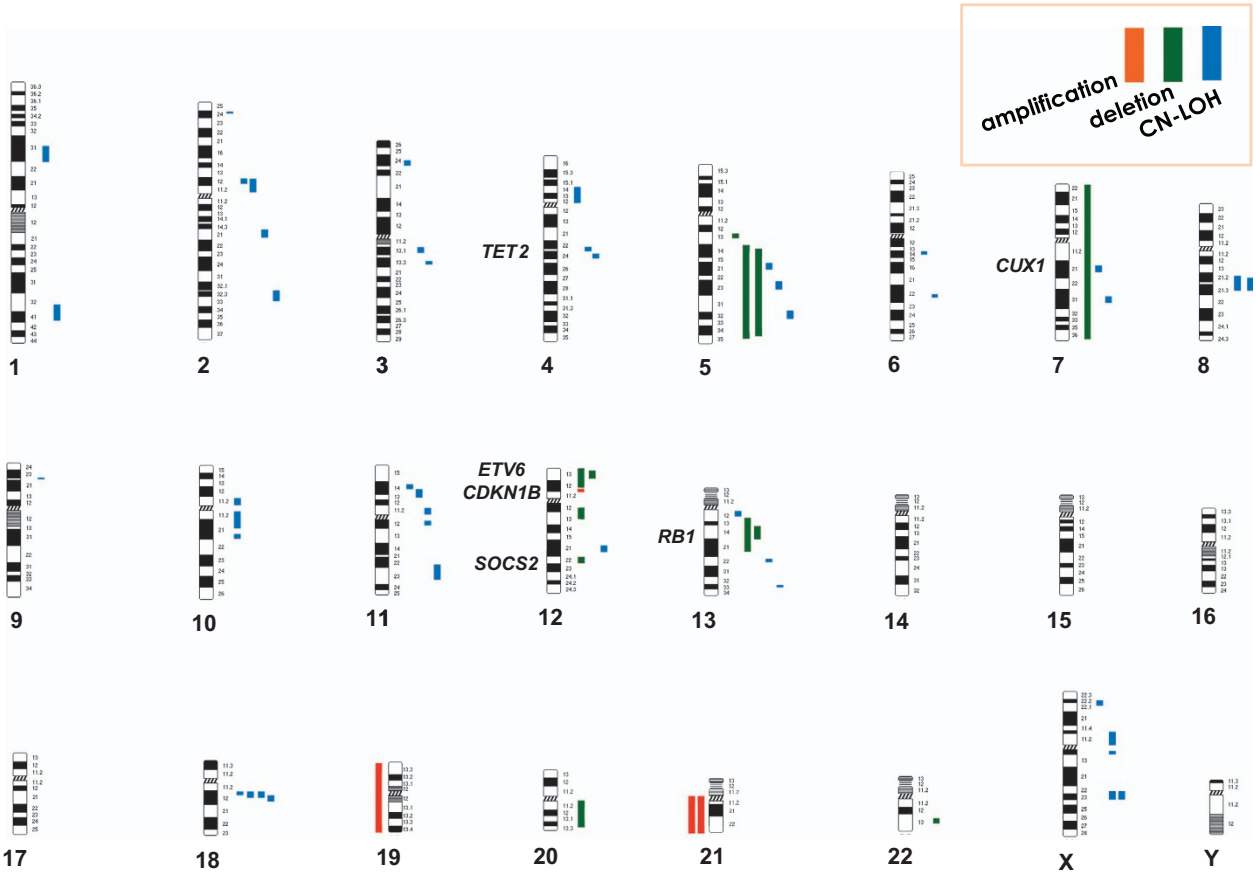

Figure 3 Regions of cytogenetic aberrations in triple-negative myeloproliferative neoplasm with overt myelofibrosis. The red bar indicates specific regions of amplification, the green bar indicates deletion, and the blue bar represents regions of neutral-loss of heterozygosity (CN-LOH) for each patient. Some genes known or suspected to play a role in leukemogenesis are indicated.

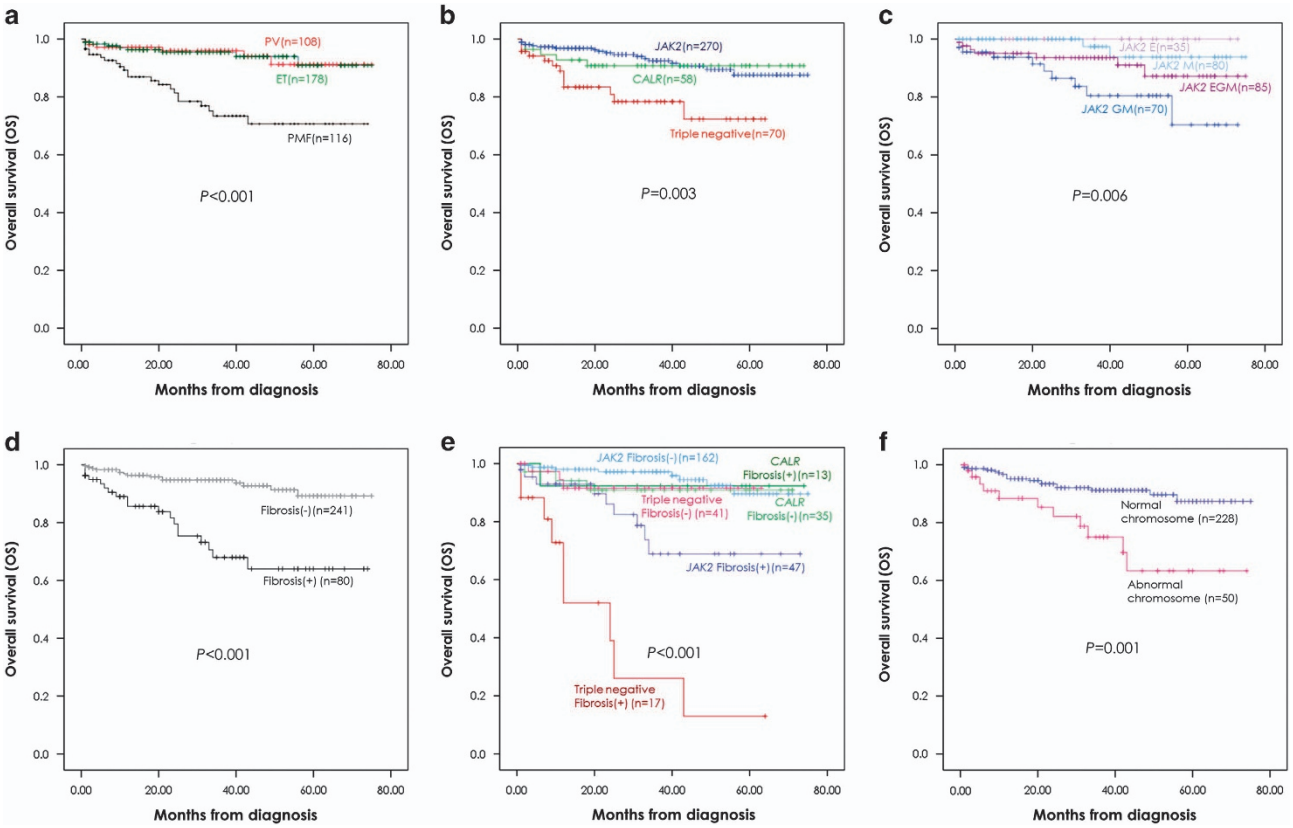

Figure 4 Overall survival in the entire patient group. WHO classification (**a**), driver mutation (**b**), lineage hyperplasia in *JAK2*-mutated patients (**c**), bone marrow fibrosis (**d**), driver mutation and bone marrow fibrosis (**e**) and chromosomal abnormalities (**f**) (E, erythroid; ET, essential thrombocythemia; fibrosis (−), grade <2; fibrosis (+), grade ⩾2; G, granulocytic; M, megakaryocytic hyperplasia; PMF, primary myelofibrosis; PV, polycythemia vera).

mutation was present as a minor clone. Except for these rare cases, *JAK2*, *CALR*, and *MPL* mutations are mutually exclusive and are considered to be driver mutations for three distinct clonal MPNs.[8,10,30]

The driver mutation influenced the laboratory findings and clinical presentation of MPNs, including age of onset, leukocyte count, red blood cell count and platelet count. When compared with patients with the *JAK2*-mutation, *CALR*-mutated MPN patients were younger and had a higher platelet count, lower leukocyte count and lower red blood cell count. The major thrombosis events did not occur in *CALR*-mutated patients although the median platelet count was highest in that group. These results confirm and extend previous data.[5,16,31–33]

BM pathology remains the central diagnostic platform for MPNs. BM cellularity, proliferation lineage, the morphology of megakaryocytes and the presence of BM fibrosis provide pathognomonic clues in the WHO classification. Among these clues, proliferation lineage and BM fibrosis were representative pathologic features with strong interobserver reliability.[34] Our study demonstrated that the driver mutation directed the proliferation lineage of the BM. *JAK2*-mutated MPN showed erythroid, granulocytic and/or megakaryocytic hyperplasia whereas *CALR*- and *MPL*-mutated MPNs displayed granulocytic and/or megakaryocytic hyperplasia.[6,35] In *JAK2*-mutated MPNs, patients with the exon 12 mutation always showed erythroid hyperplasia. We used a fragment analysis-based assay to detect exon 12 mutations, which is the first attempt to measure the allele burden. The median mutant allele burden in exon 12 mutations was lower than the burden of V617F (43.5% compared with 85.3%, $P<0.001$). These findings support previous findings demonstrating that the homozygous clone is rare in exon 12 mutations whereas it is more dominant in V617F.[36,37] The mutant allele burden was also associated with lineage hyperplasia and peripheral cytosis. *JAK2*-GM revealed a higher mutant allele burden and higher leukocyte count than *JAK2*-M, indicating that the increment of mutant allele burden potentiated the clonal proliferation of granulocytic lineage. *CALR* mutation was predominantly associated with marked megakaryocytic hyperplasia. In *CALR*-mutated MPNs, two common variants composed more than 80% of the *CALR* mutations. In this study, the type 1 and type 2 variants accounted for 62.5% and 30.4% of *CALR*-mutant patients, respectively. Frequencies of these same mutation variants were reported to be type 1 in 45–53% and type 2 in 32%–41%.[5,6] The type 2 variant occurred more frequently in ET than in PMF and revealed high platelet counts at diagnosis.[38] Our study also showed that the type 2 variant was associated with a higher platelet count.

BM fibrosis is significant not only because it is most likely the key parameter for differentiating between ET and PMF but

also because BM fibrosis represents disease progression.[13,14] This study revealed that BM fibrosis, the mutant allele burden and cytogenetic aberrations are related to one another. Patients with overt fibrosis showed a higher mutant allele burden and a higher incidence of chromosomal abnormalities than patients without overt fibrosis. These findings are consistent with the results from a previous study showing that the *JAK2* mutant allele burden is associated with progression into post-ET and post-PV myelofibrosis and that the *CALR* mutant allele burden is also higher in post-ET myelofibrosis than ET.[14,16] The increase in chromosomal abnormalities was also associated with disease progression.[17,39]

Notably, survival analyses revealed that a driver mutation and BM fibrosis were associated with clinical outcomes. Tripe-negative MPNs with overt fibrosis revealed the poorest OS, followed by *JAK2*-mutated MPNs with overt fibrosis. A genome-wide array study demonstrated that genomic alterations, including amplification, deletion and CN-LOH, were observed in all triple-negative MPNs with overt fibrosis. Loss of 5q and 13q as well as known potential leukemogenic genes including *TET2*, *CUX1*, *ETV6*, *CDKN1*, *RB1* and *SOCS2* were identified in this study. CN-LOH was the most commonly detected genomic alteration (84.6%) that was not observable in conventional chromosomal analysis. Although constitutional CN-LOH was not clearly separated from somatic CN-LOH, constitutional CN-LOH was observed less frequently in healthy controls and in other hematologic malignancies of the same ethnic population.[40,41] In addition, forms of constitutional LOH have been implicated in a predisposition to malignancies.[42] These results provide basic information to genetically characterize the triple-negative MPNs and may be productively exploited to determine the true pathogenic significance of genomic alterations. The prognostic significance of the driver mutation and BM fibrosis suggests the necessity of a prospective therapeutic strategy including kinase inhibitors and hematopoietic stem cell transplantation to enhance clinical outcomes.

In summary, this study demonstrated the driver-mutation-directed pathologic features of MPNs as well as laboratory findings and clinical presentation. Genetic–pathologic characteristics including mutant allele burden, cytogenetic aberrations, proliferation lineage and BM fibrosis provided the information for understanding the disease pathogenesis and the progression of MPNs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)