*MPL*515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients

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Recently, a gain-of-function *MPL* mutation, *MPL*W515L, was described in patients with *JAK2*V617F-negative myelofibrosis with myeloid metaplasia (MMM). To gain more information on mutational frequency, disease specificity, and clinical correlates, genomic DNA from 1182 patients with myeloproliferative and other myeloid disorders and 64 healthy controls was screened for *MPL*515 mutations, regardless of *JAK2*V617F mutational status: 290 with MMM, 242 with polycythemia vera, 318 with essential thrombocythemia (ET), 88 with myelodysplastic syndrome, 118 with chronic myelomonocytic leukemia, and 126 with acute myeloid leukemia (AML). *MPL*515 mutations, either *MPL*W515L (n = 17) or a previously undescribed *MPL*W515K (n = 5), were detected in 20 patients. The diagnosis of patients with mutant *MPL* alleles at the time of molecular testing was de novo MMM in 12 patients, ET in 4, post-ET MMM in 1, and MMM in blast crisis in 3. Six patients carried the *MPL*W515L and *JAK2*V617F alleles concurrently. We conclude that *MPL*W515L or *MPL*W515K mutations are present in patients with MMM or ET at a frequency of approximately 5% and 1%, respectively, but are not observed in patients with polycythemia vera (PV) or other myeloid disorders. Furthermore, *MPL* mutations may occur concurrently with the *JAK2*V617F mutation, suggesting that these alleles may have functional complementation in myeloproliferative disease. (Blood. 2006;108:3472-3476)

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Introduction

The identification of *JAK2*V617F in virtually all patients with polycythemia vera (PV) and in 50% to 75% of those with either essential thrombocythemia (ET) or de novo myelofibrosis with myeloid metaplasia (MMM) has shed new light on the molecular pathogenesis of these disorders but has also raised important questions in this regard. *JAK2*V617F is a somatic mutation that constitutively activates the JAK2 tyrosine kinase,[1-3] which normally plays a critical, nonredundant role in mediating signal transduction downstream of several cytokine receptors and is indispensable for definitive erythropoiesis and normal myeloid lineage differentiation.[4,5] *JAK2*V617F confers cytokine hypersensitivity[1-3] and has been demonstrated in erythropoietin-independent erythroid colonies (EECs) derived from PV and ET patients.[6] Furthermore, *JAK2*V617F expression in murine bone marrow transplant models results in a type of myeloproliferative disease (MPD) that resembles PV, albeit with important strain-related phenotypic differences.[1,7]

Several lines of evidence, however, have supported the existence of mutations other than *JAK2*V617F in MPD. For example, studies of familial MPD, including familial PV, indicate that *JAK2*V617F may be acquired secondarily, possibly as a "patterning" mutation, in a manner that may be independent of disease duration.[8-11] Furthermore, the phenotypic pleiotropy associated with the acquisition of *JAK2*V617F and the variable burden of the mutant allele in MPD raise the question of whether *JAK2*V617F is sufficient for MPD pathogenesis. In addition, recent studies using X-chromosome inactivation pattern analysis demonstrated that most patients with *JAK2*V617F-negative ET and MMM exhibit clonal hematopoiesis, thus pointing to the presence of an alternative disease-promoting mutation.[12-14] Furthermore, quantitation of the *JAK2*V617F allele in one of these studies indicated that in some MPD patients, only a fraction of clonally derived granulocytes harbored the mutant *JAK2* allele, suggesting not only that *JAK2*V617F was acquired secondarily but that some MPD patients acquire more than one mutation during disease progression.

This possibility has been borne out by the recent identification of the *MPL*W515L mutation in 4 of 45 *JAK2*V617F-negative MMM patients.[15] As is *JAK2*V617F, *MPL*W515L is an acquired mutation that induces constitutive, cytokine-independent activation of the JAK-STAT pathway. Furthermore, the expression of *MPL*W515L in murine bone marrow resulted in an MPD phenotype that recapitulated certain clinical and histopathologic features

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of MMM and ET. Several questions, however, remain unanswered: Are *MPL*W515L and *JAK2*V617F mutually exclusive mutations? What is the prevalence and disease distribution (phenotype) of *MPL*W515 mutations? Are *MPL*515 mutations clinically important? These questions are addressed in the current study through a bi-institutional study of a large cohort of patients with a spectrum of myeloid disorders. In future studies, identification of affected patients in a prospective fashion should enable additional studies that examine clonal distribution, gene expression profiling, clonal evolution over time, and drug sensitivity of primary cells to JAK2 inhibitors.

Patients, materials, and methods

Sample collection and processing

The current study was approved by the institutional review boards of both the Mayo Clinic and the Dana Farber Cancer Institute. All patients provided informed written consent for study sample collection and permission for research use. Peripheral blood (PB) and bone marrow (BM) study samples were accrued from patients seen in the Mayo Clinic MPD practice or from participants in the Harvard Myeloproliferative Disorders Study.[2] DNA was prepared from granulocyte PB mononuclear cells (PBMNCs) or archived BM cell pellets, as previously described.[16] In general, neutrophil DNA was used in all patients with MMM and PV and in approximately half of those with ET. BM cells were used in the remainder of the ET patients and for those with AML, MDS, and CMML. Diagnoses for all specific disease categories were determined according to criteria set by the World Health Organization–sponsored committee for the classification of hematologic malignancies,[17] and molecular testing was performed on tissue samples collected from patients at variable times after the initial diagnosis.

Genotyping of *MPL*515 allele

Genotyping of the initial 403 Mayo specimens was performed with the use of a LightCycler (Roche Applied Bioscience, Indianapolis, IN) assay we developed and by direct DNA sequencing. Once the LightCycler (Roche Applied Bioscience) assay was validated, only specimens (of the subsequent 396 Mayo Clinic specimens, including those of 64 healthy controls) flagged as positive were sequenced to confirm the presence of the mutation. All 447 Harvard samples were screened by DNA sequencing alone. Primers and probes were designed and selected with the use of LightCycler Probe Design software 2.0 (Roche Applied Bioscience). Each 20-μL reaction mix contained 4 μL LC FastStart DNA Master^{PLUS} HybProbe master mix, 150 ng DNA template, 0.2 μM sensor probe (5′-3′) (CTgCCACCTCAgCAgCA-LCRed640) and 0.2 μM anchor probe (5′-3′) (fluorescein-AggCCCAg-gACggCg-P), 1 μM forward primer (5′-TgggCCgAAgTCTgACCCTTT-3′), and 0.5 μM reverse primer (5′-ACAgAgCgAACCAAgAATgCCTgT-3′). The following PCR protocol was used: denaturation at 95°C for 10 minutes and amplification (95°C for 10 seconds, 57°C for 10 seconds, and 72°C for 15 seconds) for 40 cycles. Melting-curve analysis was performed as follows: melt product at 95°C, hold at 45°C for 30 seconds, then ramp to 85°C at a slope of 0.2°/sec. The sensor probe was designed to be a perfect match for the wild-type MPL sequence. This probe dissociates at 54°C when bound to mismatched sequence (W515L) and at 64°C when bound to the perfectly matched wild-type sequence (Figure 1A). PCR products were purified (QIAGEN PCR Purification Kit, Valencia, CA) and subjected to bidirectional sequence analysis on the ABI PRISM 3730 DNA Analyzer (Applied Biosystems) using the following primers: forward, 5′-ggTgACCgCTCTgCA TCTAgTgCT-3′; reverse, 5′-CACCTggTCCACCgCCAgTCT-3′.

Sensitivity of *MPL*515 LightCycler assay

DNA from a healthy control (homozygous for *MPL*515-WT) and from a patient homozygous for *MPL*W515L was mixed in various proportions to estimate assay sensitivity for mutant allele detection. Serial dilutions showed the assay sensitivity to be 3% to 5% (ie, mutant allele burden greater than 3%-5% was reproducibly detected; data not shown).

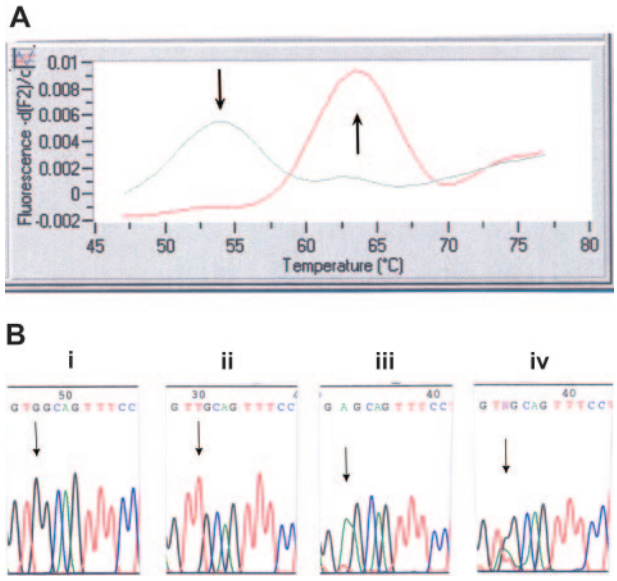

Figure 1. Mutation screening for *MPL*W515L/K. (A) LightCycler assay for *MPL*W515L. Melting curve analysis displays that probe bound to a mismatch dissociates at 54°C, whereas probe bound across the wild-type sequence dissociates at 64°C. (B) DNA chromatogram illustrating wild-type *MPL*515 sequence (i), the TGG→TTG conversion in *MPL*W515L (ii), the TGG→AAG conversion in *MPL*W515K (iii), and the presence of all 3 alleles (ie, wild-type *MPL*, *MPL*W515L, and *MPL*W515K) in 2 patients (iv).

Genotyping of *JAK2*V617F allele

Genotyping was performed by direct DNA sequencing (Mayo Clinic specimens), as previously described.[18] *JAK2*V617F mutational status was confirmed in all *MPL*515 mutation–positive samples with a previously described allele-specific PCR sensitive to 0.01% to 0.1%.[18,19] Genotyping of the Harvard specimens for JAK2V67F was performed by mass spectrometry and quantitative real-time PCR assays, as previously described.[2,12]

Results

Samples from 1182 patients and 64 healthy controls were screened for the presence of *MPL*515 mutations; 735 samples were screened at the Mayo Clinic, and 447 samples were screened at Harvard (Table 1). Overall, 20 patients were found to carry *MPL*515 mutations (Table 2). Of these, 4 MMM patients, all from the Harvard cohort carrying *MPL*W515L, have been previously reported.[15] Seventeen patients carried the previously reported *MPL*W515L mutation (Figure 1A-B), and 5 patients exhibited a previously undescribed mutation involving the same codon (*MPL*W515K) (Figure 1B; Table 2). Surprisingly, DNA sequencing showed all 3 alleles (wild-type *MPL*, *MPL*W515L, *MPL*W515K) present in 2 patients (patients 8 and 9 in Table 2; both de novo MMM), pointing to a mixed clonal state (Figure 1B). Study of 2 consecutively obtained archived BM specimens (patient 8) showed the presence of both *MPL* mutations at low levels (5%-10% of wild-type allele) in the initial specimen. The burden of both *MPL* mutant alleles was increased (40% of wild-type allele) in the subsequent specimen (collected 6 months later; data not shown). Although these data support the coexistence of *MPL*515L and *MPL*515K alleles, the possibility of sample contamination cannot be definitively excluded; hence, we consider this observation to be preliminary and subject to confirmation in future studies. Six of the

Table 1. Diagnosis and *JAK2*V617F mutational status of 1182 patients with a spectrum of myeloid disorders

		Mayo patients			Harvard patients		
	N	No. of patients	*JAK2*V617F positive, no. (%)	*MPL*515L or −515K positive, no. (%)	No. of patients	*JAK2*V617F positive, no. (%)	*MPL*515L or −515K positive, no. (%)
All patients	1182	735	252	13	447	335	7
MMM	290	198	106 (54)	8 (4)	92	46 (50)	5 (5)
De novo	NA	159	75	8	NA	NA	4
After ET	NA	10	8	0	NA	NA	1
After PV	NA	29	23	0	NA	NA	n/a
ET	318	167	76 (46)	2 (1)	151	106 (70)	2 (1)
PV	242	38	38 (100)	0	204	183 (90)	0
AML	126	126	29 (23)	3 (2)	0	n/a	n/a
With antecedent MPD	NA	37	23	3	0	n/a	n/a
MDS	88	88	0	0	0	n/a	n/a
CMML	118	118	3 (3)	0	0	n/a	n/a

MMM indicates myelofibrosis with myeloid metaplasia; ET, essential thrombocythemia; PV, polycythemia vera; AML, acute myeloid leukemia; MPD, myeloproliferative disorder; MDS, myelodysplastic syndrome; CMML, chronic myelomonocytic leukemia; NA, accurate subclassification not available because of study design; and n/a, not applicable.

20 (30%) patients carried both *MPL*W515L and *JAK2*V617F mutations—2 ET, 3 de novo MMM, and 1 post-ET MMM. *MPL*515 mutations were restricted to patients with a history of either MMM or ET; specific diagnosis at the time of molecular testing was de novo MMM in 12 patients, ET in 4 patients, post-ET MMM in 1 patient, and MMM in blast crisis in 3 patients (Table 2). Clinical and laboratory features of the 20 patients affected by *MPL* mutations (11 males; median age, 58.5 years; median follow-up, 67 months) are presented in Table 2.

Among the 13 Mayo Clinic patients with *MPL* mutations, 11 had a history of MMM, 2 had a history of ET, and none had a history of familial MPD (Table 2). Among the former, 3 were in blast crisis at the time of molecular testing (2 AML-M2, 1 AML-M0) and one transformed to unclassified AML within 1 year of testing. Another patient had advanced, treatment-refractory disease with severe bone pain, progressed to accelerated-phase disease with 15% circulating blasts, and died within 2 years of the study time point. Two patients were known to have had chronic thrombocytosis before the diagnosis of de novo MMM, and each has had a stable postdiagnosis clinical course for 3 and 5 years, respectively. Similarly, 2 other patients with intermediate risk disease[20] showed slow progression after 2 and 4 years from the time of molecular testing. The last 2 patients with MMM presented with high-risk disease and have been followed up for less than 2 years from the time of testing. Of the 2 Mayo Clinic patients with *MPL* mutations and ET, one has had a 14-year disease duration with stable clinical course, while the other patient died of AML after 14 years of disease and after having received treatment with hydroxyurea, busulfan, radiophosphorus, and anagrelide. Review of bone marrow histology and cytogenetic data did not disclose unexpected findings in patients with *MPL* mutations. Because of the method of patient sample accrual (see "Patients, materials, and methods"), clinical details for the 7 patients from the Harvard study were limited; 5 carried the diagnosis of MMM (4 de novo MMM, 1 post-ET MMM) and 2 had ET (Table 2). Thrombohemorrhagic complications were not a prominent feature of the disease in any of the aforementioned patients from the Mayo Clinic or Harvard.

Discussion

The initial identification of a gain-of-function *MPL* mutation (*MPL*W515L) provided proof-of-principle for a novel mechanism of constitutive JAK-STAT signaling in acquired MPD lacking the *JAK2*V617F mutation.[15] In a substantially expanded analysis of 1182 patients with a spectrum of acute and chronic myeloid disorders, we found 20 patients carrying *MPL*515 mutations, indicating that this is a relatively infrequent event. A significant proportion (25%) of the 20 patients carried a previously undescribed mutation, *MPL*W515K. Although further analysis of the MPLW515K allele is necessary, it seems likely that it is a gain-of-function mutation and points to the *MPL* codon 515 as a hot spot for activating mutations in acquired MPD. These findings are also consistent with the observation that the deletion of the amphipathic KWQFP motif in the transmembrane-cytoplasmic hinge region of murine or human MPL (underlined residue corresponds to W515) constitutively activates downstream signaling in a ligand-independent fashion.[21] The previous identification of a highly penetrant gain-of-function *MPL* mutation in the transmembrane (TM) domain (*MPL*S505N) in a Japanese pedigree with familial ET[22] and the spontaneously occurring *MPL*W508S mutation in murine *MPL* (corresponds to W515S in human MPL)[23] further support the critical role of the TM and membrane-distal domains in regulating MPL function.

Although *MPL*W515 mutations are infrequent in MPD, valuable insights can be gleaned about the relationship between mutant alleles in the JAK-STAT pathway and phenotypic manifestations of disease. First, we observed *MPL*W515 mutations only in patients with ET or MMM, never in patients with PV. This suggests that *MPL*W515 mutations may favor megakaryocytic lineage fate determination as opposed to erythroid fates. In contrast, the almost invariant presence of the *JAK2*V617F allele in patients with PV and the high frequency of PV patients with duplication of the *JAK2*V617F allele compared with patients with ET or MMM suggest that the *JAK2*V617F allele favors erythroid fate determination in hematopoietic progenitors. The hypothesis that MPL mutation favors megakaryocytic fate but that *JAK2*V617F favors erythroid fate is supported by observations in murine models of disease, in which overexpression of *JAK2*V617F results in a PV-like phenotype without thrombocytosis,[1,15] whereas the expression of *MPL*W515L in a murine model results in a phenotype characterized by marked thrombocytosis.[15] The *JAK2*V617F allele is present in the hematopoietic stem cell compartment in humans,[24] and this observation supports the notion that the mutant allele must have some instructive role in the phenotypic manifestation of the diseases. Perhaps of most interest, 6 patients had both the

Table 2. Clinical, laboratory and molecular characteristics of patients with *MPL*515 mutations (at the time of MPL515 testing)

Patient no.	Sex/age, y, at diagnosis	Diagnosis	*JAK2*V617F status	*MPL*515 status	*MPL*515 mutant peak/wild-type peak ratio, %	Cytogenetics	Hgb, g/dL	WBC count, $\times 10^9$/L	Platelet count, $\times 10^9$/L	AML	Status at last follow-up	Duration of follow-up, mo
Mayo study												
1	M/32	De novo MMM	WT	W515L	< 50	Normal	11.4	5.5	436	No	Alive	40
2	M/46	De novo MMM	Heterozygous	W515L	< 50	del(13q)	9.5	5.3	379	No	Deceased	91
3	M/64	De novo MMM	WT	W515L	< 50	del(20q)	9.9	5.7	182	No	Alive	94
4	M/60	De novo MMM	WT	W515K	< 50	Normal	8.3	4.5	215	No	Alive	30
5	M/61	De novo MMM	WT	W515L	< 50	ND	11.5	36.6	139	No	Alive	22
6	M/75	Post–de novo MMM AML (M0)	WT	W515L	> 50	t(1;7) t(1;21)*	9.0	72.1	161	n/a	Deceased	34
7	F/52	Post–de novo MMM AML (M2)	WT	W515K	100	Normal	9.0	10.6	430	n/a	Alive	67
8	M/49	De novo MMM	Heterozygous	W515L + W515K	< 50	Normal	8.2	4.6	40	No	Alive	23
9	F/53	De novo MMM	Heterozygous	W515L + W515K	< 50	Complex abnormalities	6.3	8.5	124	Yes	Deceased	20
10	M/58	De novo MMM	WT	W515K	100	del(11q)*	11.5	10.3	146	No	Alive	108
11	M/71	ET	WT	W515L	< 50	Normal	14.0	6.9	959	No	Alive	168
12	F/67	ET	WT	W515L	> 50	t(1;21)	9.1	4.1	1539	Yes	Deceased	172
13	M/81	Post-ET MMM AML (M2)	WT	W515L	100	t(8;21)	9.6	47.4	161	n/a	Deceased	72
Harvard study												
14	F/59	De novo MMM	WT	W515L	NA	Normal	NA	NA	NA	No	Alive	NA
15	F/5	De novo MMM	WT	W515L	NA	Normal	9.4	7.5	261	No	Alive	NA
16	M/58	De novo MMM	WT	W515L	NA	del(16q)*	10.8	7.2	120	Yes	Deceased	36
17	F/68	De novo MMM	WT	W515L	NA	Normal	10.1	17.7	559	No	Alive	96
18	F/45	ET	Heterozygous	W515L	NA	Normal	NA	NA	NA	No	Alive	24
19	F/56	ET	Heterozygous	W515L	NA	Normal	NA	NA	658	No	Alive	168
20	F/67	Post-ET MMM	Heterozygous	W515L	NA	Normal	NA	NA	650	No	Alive	NA

*MPL*515 mutant peak/wild-type peak ratio was the ratio on sequencing chromatogram ×100. MMM indicates myelofibrosis with myeloid metaplasia; ET, essential thrombocythemia; AML, acute myeloid leukemia; WT, wild type; Hgb, hemoglobin; n/a, not applicable; NA, not available; and ND, not done.

*Cytogenetic abnormalities developed after identification of *MPL*515 mutations.

*MPL*W515L and the *JAK2*V617F mutations. In all cases, the *MPL*515 mutant allele was present in excess of the *JAK2*V617F allele. Although the former was detectable by a low-sensitivity screening method (DNA sequencing) (*MPL* 515L or 515K/*MPL* 515W peak ratio is greater than 50% in 5 of 13 Mayo Clinic patients) (Table 2), the *JAK2*V617F allele was detectable only by the highly sensitive allele-specific PCR assay. From a pathogenetic standpoint, our current experimental approach does not distinguish between the 2 possibilities regarding whether *MPL* and *JAK2* mutations arise in independent clones or whether the *JAK2*V617F-bearing cell emerges as a subclone of the *MPL* mutation–carrying progenitor cell. Patients with both *MPL*W515L and *JAK2*V617F had a diagnosis of ET or MMM, suggesting a potent effect of *MPL*W515L in supporting megakaryocytic hyperplasia and thrombocytosis, but it will be of interest to determine whether this subset of ET patients has a higher average hemoglobin level that those with *MPL*W515L alone. Further studies to characterize the mutations in prospectively purified hematopoietic progenitors and murine modeling will be necessary to further clarify this issue.

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