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ORIGINAL ARTICLE

Screening for *MPL* mutations in essential thrombocythemia and primary myelofibrosis: normal Mpl expression and absence of constitutive STAT3 and STAT5 activation in *MPL*W515L-positive platelets

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Abstract

Objectives: To evaluate the frequency of *MPL* W515L, W515K and S505N mutations in essential thrombocythemia (ET) and primary myelofibrosis (PMF) and to determine whether *MPL*W515L leads to impaired Mpl expression, constitutive STAT3 and STAT5 activation and enhanced response to thrombopoietin (TPO). *Methods:* Mutation detection was performed by allele-specific PCR and sequencing. Platelet Mpl expression was evaluated by flow cytometry, immunoblotting and real-time RT-PCR. Activation of STAT3 and STAT5 before and after stimulation with increasing concentrations of TPO was studied by immunoblotting. Plasma TPO was measured by ELISA. *Results:* *MPL*W515L was detected in 1 of 100 patients with ET and 1 of 11 with PMF. Platelets from the PMF patient showed 100% mutant allele, which was <50% in platelets from the ET patient, who also showed the mutation in granulocytes, monocytes and B cells. Mpl surface and total protein expression were normal, and TPO levels were mildly increased in the *MPL*W515L-positive ET patient, while *MPL* transcripts did not differ from controls in both *MPL*W515L-positive patients. Constitutive STAT3 and STAT5 phosphorylation was absent and dose response to TPO-induced phosphorylation was not enhanced. *Conclusions:* The low frequency of *MPL* mutations in this cohort is in agreement with previous studies. The finding of normal Mpl levels in *MPL*W515L-positive platelets indicates this mutation does not lead to dysregulated Mpl expression, as frequently shown for myeloproliferative neoplasms. The lack of spontaneous STAT3 and STAT5 activation and the normal response to TPO is unexpected as *MPL*W515L leads to constitutive receptor activation and hypersensitivity to TPO in experimental models.

Key words Mpl; essential thrombocythemia; primary myelofibrosis; myeloproliferative neoplasms; STAT3; STAT5; *MPL*W515L; *MPL*W515K; *MPL*S505N

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The identification of the *JAK2*V617F mutation has contributed significantly to the understanding of the molecular pathogenesis of myeloproliferative neoplasms (MPN). However, the underlying genetic defects remain unknown in approximately half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1). The detection of *MPL* somatic mutations has begun to unravel the pathogenesis of *JAK2*V617F-negative patients (2). Several point mutations have been identified in *MPL* exon 10, most of them involving codon 515, which is located in an amphipathic juxtamembrane motif (RWQFP) that prevents autonomous receptor activation (3) and include W515L and W515K (2, 4). Mutations in this motif are believed to activate Mpl by allowing the

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receptor to assume an active dimeric conformation in the absence of thrombopoietin (TPO) binding (5), although the precise molecular mechanisms have yet to be clarified. Other acquired mutations are located in codon 505 and result in S505N (6), which has been previously described in a pedigree with familial thrombocythemia (7). Overall frequency of *MPL* mutations ranges from 1% to 4% for ET (4, 6, 8–11) and 5% to 11% for PMF patients (4, 6, 12). These mutations are more frequent in *JAK2*V617F-negative patients (6, 13), although coexistence of both mutant alleles has been described (2, 8). The *MPL*W515L mutation has also been detected in other myeloid neoplasms, such as refractory anemia with ringed sideroblasts and thrombocytosis (14) and in 25% of patients with primary and secondary acute megakaryoblastic leukemia (15). Murine models reveal that *MPL*W515L-transduced animals develop an aggressive MPN characterized by marked thrombocytosis, leukocytosis and bone marrow fibrosis with extramedullary hematopoiesis (2). Overexpression of these mutant alleles in cell lines leads to cytokine-independent growth and constitutive activation of the JAK/STAT pathway. In addition, MPLW515L-expressing cell lines display hypersensitivity to TPO, as assessed by cell growth and phosphorylation of downstream signaling molecules (2). However, the precise functional effects of these mutations in signaling and TPO-response in patient samples have not been explored.

Decreased Mpl expression is a frequent finding in MPN patients (16, 17), although the frequency of this defect varies according to disease phenotype, involving most patients with polycythemia vera and PMF (18), while a variable proportion of ET patients show this abnormality (16, 18–20). The mechanisms underlying this defect remain unclear. Potential explanations include abnormal receptor maturation and trafficking (21), decreased gene expression (16) or down-modulation triggered by increased internalization (22). Although an inverse relationship has been found between *JAK2*V617F allele burden and Mpl levels, impaired Mpl expression has also been observed in the absence of *JAK2*V617F (18), suggesting that alternative molecular defects may lead to the same phenotypic abnormality. The pattern of Mpl expression in patients with exon 10 *MPL* mutations has not been investigated. It is plausible that these mutations might affect receptor synthesis or stability or, alternatively, lead to tyrosine phosphorylation of the cytoplasmic domain and subsequent internalization (23), as shown for patients with familial thrombocythemia caused by mutations in the *THPO* gene, in whom excessive stimulation of Mpl triggers receptor down-modulation (24, 25).

The aim of this study was to analyze the frequency of *MPL*W515L, *MPL*W515K and *MPL*S505N mutations in a cohort of patients with ET and PMF and to evaluate the effects of *MPL*W515L in Mpl expression and signaling pathways in patients positive for this mutation.

Patients and methods

Patients

One hundred consecutive patients with ET and eleven with PMF were included in this study. Clinical and laboratory features were evaluated retrospectively by chart review. Diagnosis was made according to the Polycythemia Vera Study Group or the Italian Consensus Conference criteria for ET and PMF, respectively. This study was approved by IDIM A. Lanari Institutional Ethics Committee, and patients signed an informed consent, in accordance with the declaration of Helsinki. Age at diagnosis was 44 (9–81) yr old, 81 were women. Time from diagnosis to sample collection was 69 (1–222) months, and overall disease duration was 94.5 (7–273) months. Prior therapy included anagrelide in 30 patients, hydroxyurea in 19, thalidomide plus prednisone in one and more than one agent, which were sequentially administered and included anagrelide, hydroxyurea and/or interferon, in 29.

Blood samples

DNA was prepared by standard procedures from peripheral blood leukocytes. To prepare platelet RNA, platelet-rich plasma (PRP) was obtained by centrifugation at 200g for 8 min and supplemented with 0.042 M indomethacin. To remove leukocytes and red cells, PRP was subjected to an additional centrifugation step at 200 g during 8 min and filtered through a leukocyte reduction filter (Purecell PL, Pall Biomedical Products Co, NY, USA). After this procedure, the leukocyte:platelet ratio was $< 1 : 10^6$. Total RNA was prepared from 5×10^9 platelets, and 1 μg RNA was reverse transcribed using SuperScript (Life Technologies, Carlsbad, CA, USA). To study lineage-specific involvement, peripheral blood was layered over a Ficoll-Hypaque density gradient. Monocytes ($CD14^+$), B cells ($CD19^+$) and T cells ($CD3^+$) were separated from the mononuclear cell low-density fraction by flow cytometric cell sorting in a FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA), and granulocytes were recovered from the upper interphase of the bottom layer after red cell lysis. Cell populations had a purity of 98% or greater and were used for DNA extraction.

Genotyping for *MPL* exon 10 and *JAK2*V617F mutations

Mutation detection was performed in leukocyte DNA in 93 patients, and in platelet cDNA in 18 patients.

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Allele-specific PCR for *MPL*W515L, W515K and S505N were performed in DNA according to Beer *et al.* (6). Assay sensitivity was calculated by mixing DNA carrying known percentages of mutant alleles, kindly provided by P. Beer, University of Cambridge, UK, with normal DNA, and was 1% for *MPL*W515L, 2% for *MPL*W515K and 5% for *MPL*S505N. For mutation detection in platelet cDNA, the following flanking primers were used: forward out (FO) 5′- CGATCTCGCT-ACCGTTTACAG- 3′, reverse out (RO) 5′-GGGAG-GATTTCAAGGAGGC- 3′ with the same inner mutant primers used for DNA. Levels of *MPL*W515L relative to wild-type allele were assessed by real-time PCR on a iCycler (Bio Rad Life Science, Hercules, CA, USA) by amplifying the mutant and wild-type alleles in two separate tubes, using mutant (5′-CCTGCTGCTGCTGA GGTT-3′) and RO primers in the first tube and wild-type (5′-TAGTGTGCAGGAAACTGCCA-3′) and FO primers in the second, and IQ™ SYBR Green Supermix (Bio Rad Life Science). A curve was obtained by plotting ΔC_T (C_T mutant – C_T wild-type) respect to serial dilutions of wild-type cDNA into a sample carrying 100% *MPL*W515L. Determination of *JAK2*V617F was performed by allele-specific PCR, with an assay sensitivity of 1–2% (26). *MPL* exon 10 sequencing was performed after amplification with FO and RO primers, product purification and sequencing in both directions on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Platelet surface Mpl expression by flow cytometry

PRP was prepared by centrifugation at 200 g for 8 min and 1×10^6 platelets were incubated with 5 μg/mL anti-Mpl monoclonal mouse IgG (R&D Systems, Minneapolis, MN, USA) or mouse IgG as isotype control (BD Pharmingen, San Diego, CA, USA). This step was followed by incubation with a goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (BD Pharmingen) and a phycoerythrin (PE)-conjugated CD41 and analyzed by flow cytometry. A ratio between mean fluorescence intensity (MFI) obtained with the Mpl antibody and the isotypic control was calculated.

Western blot assays for Mpl, phosphoSTAT3 and phosphoSTAT5

PRP supplemented with 0.042 M indomethacin was filtered through a leukocyte reduction filter, red cells were lysed with NH_4Cl, and platelets were washed twice. For STAT3 and STAT5 phosphorylation assays, platelet samples were processed without cytokine addition for constitutive activation studies or stimulated with 0.5, 1.0, 10, 50 and 100 ng/mL recombinant human TPO (R&D Systems) at 37°C during 10 min. Samples were immediately centrifuged and resuspended in lysis buffer with the addition of an inhibitor cocktail. Total protein was separated by SDS-PAGE and transferred to nitrocelullose membranes. For analysis of Mpl content, blots were revealed with anti-Mpl monoclonal mouse IgG (R&D Systems) and reprobed with anti-β_3 integrin murine monoclonal antibody (kindly provided by Dr. T. Kunicki, The Scripps Research Institute, CA, USA). For STAT3 and STAT5 phosphorylation studies, membranes were probed with rabbit anti-phosphoTyr705-STAT-3, anti-STAT-3, anti-phosphoTyr694-STAT-5 or anti-STAT-5 (Cell Signaling, Woburn, MA, USA). After washing, membrane-bound primary antibodies were detected on autoradiographic films by horseradish peroxidase-conjugated secondary antibodies and the ECL Plus chemiluminescent system (Amersham Pharmacia Biotech, Buckinghamshire, UK), and protein bands were quantified by densitometry.

Real-time polymerase chain reaction for platelet *MPL* transcript levels

Relative expression of *MPL* in platelet cDNA was determined by real-time PCR using IQ™ SYBR Green Supermix and normalized to *GAPDH*, F and R primers for *MPL* and *GAPDH* were 5′-AGGAGACTGAGGCATG-CCCT-3′, 5′-GGCTGGGTTCCACTTCTTCA-3′, and 5′-CGACCACTTTGTCAAGCTCA-3′, 5′-CCCTGTTG-CTGTAGCCAAAT-3′, respectively. Samples were run in triplicate, and a ratio between relative amounts of *MPL* and *GAPDH* was calculated using the C_T values for each transcript.

Plasma thrombopoietin levels

Plasma was prepared at 4°C by sequential centrifugation at 2.000 g for 30 min and 10.000 g for 10 min, and TPO was measured by enzyme-linked immunosorbent assay (R&D Systems).

Statistical analysis

Comparison between RNA levels in patients and controls was performed using Mann-Whitney-Wilcoxon test. Two-sided P-values < 0.05 were considered significant.

Results

Genotyping for MPL exon 10 and JAK2V617F mutations

One of 100 patients with ET (1%) and 1 of 11 with PMF harbored the *MPL*W515L mutation by allele-spe-

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cific PCR, Fig. 1A, which was detected in both leukocytes and platelets from the ET patient, while only platelet RNA was available from the PMF patient. Lineage-specific involvement in the ET patient was studied by DNA sequencing of different cell populations, which showed the presence of *MPL*W515L in granulocytes, monocytes and B cells, while it was absent from T cells, Fig. 1B. When the T-cell fraction was subjected to allele-specific PCR, which has higher sensitivity, a faint mutant band was found (data not shown). Platelet cDNA sequencing chromatogram showed only the mutant peak in the PMF patient, while the mutant allele was estimated to be $<50\%$ in platelets from the ET patient, Fig. 1B. To compare *MPL*W515L burden in platelets from both patients, mutant relative to wild-type allele was measured by real-time PCR, and a curve was obtained by mixing dilutions of cDNA from the PMF patient, considered to harbor 100% mutant allele, into a normal control, showing that mutant allele burden in the ET patient was 38%. In this cohort, screening for *MPL*W515K and S505N mutations yielded negative results, while 49 (49%) patients with ET and 2 of 11 with PMF were *JAK2*V617F positive.

Clinical features of *MPL*W515L-positive patients revealed that the female patient with ET, who was 43 yr old at diagnosis, suffered from pulmonary embolism at age 48, when the platelet count was 900×10^9/L. She was treated with oral anticoagulants and hydroxyurea, which was subsequently replaced for anagrelide, without thrombosis recurrence, after an 8.5 -yr overall follow-up. PMF was diagnosed in the other *MPL*W515L-positive female patient at age 64. Her clinical course, which was recently described (27), was characterized by anemia and marked splenomegaly. She failed to respond to thalidomide and prednisone and died after leukemic transformation at 7-yr follow-up. Both patients were negative for *JAK2*V617F and had normal cytogenetics.

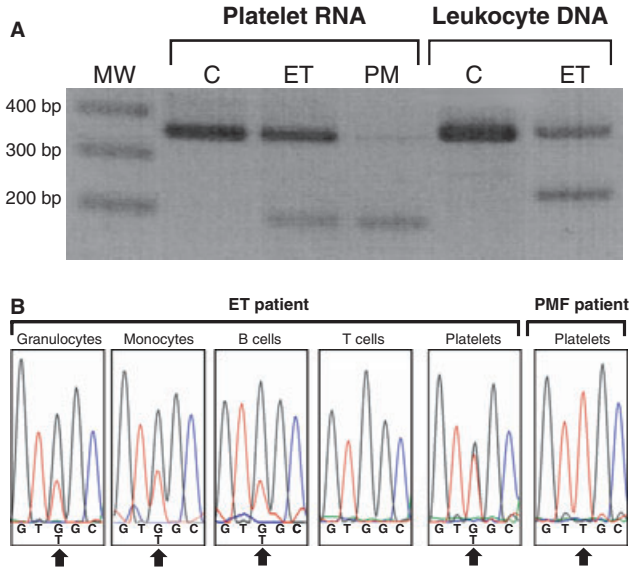

Figure 1 Detection of *MPL*W515L mutation. (A) Analysis of the *MPL*W515L mutation by allele-specific PCR. The *MPL*W515L mutation was detected in platelet RNA in one patient with essential thrombocythemia (ET) and one with primary myelofibrosis (PMF) and in leukocyte DNA from the patient with ET. Two bands (356 and 189 bp for RNA and 353 and 233 bp for DNA) indicate the presence of the mutation, while one band represents the normal genotype in the control (C). (B) Detection of the *MPL*W515L mutation by sequence analysis. Sequencing traces show the presence of a mutant T (red) peak in granulocytes, monocytes and B cells, while only the wild-type G (black) peak is evident in T cells from the ET patient. Platelet cDNA from the PMF patient shows only the mutant peak, which is <50% in platelets from the ET patient. Arrows indicate the presence of the mutation.

Surface and total platelet Mpl levels

Cell surface Mpl expression by flow cytometry was normal in the *MPL*W515L-positive ET patient compared to a simultaneously studied normal control, Mpl/isotype MFI ratio was 3.6 vs. 3.2, Fig. 2A. In accordance with cell surface expression, total platelet Mpl content was normal in this patient, Mpl/β3 integrin ratio, calculated

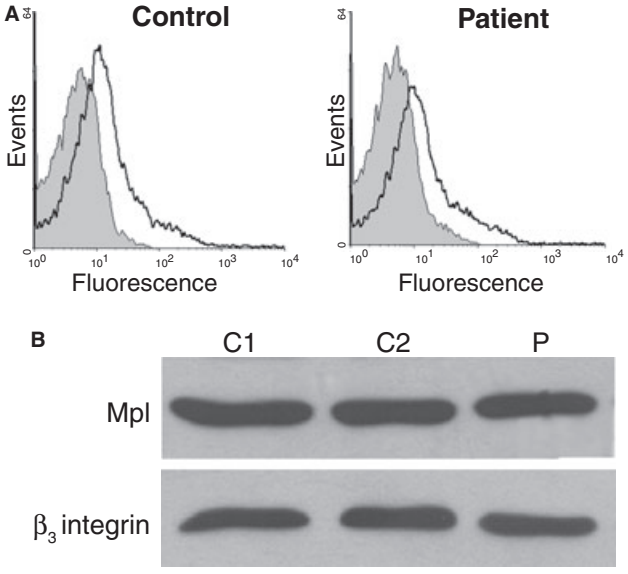

Figure 2 Mpl surface expression and total Mpl content in platelets. (A) Flow cytometry shows normal Mpl cell surface expression in platelets from the *MPL*W515L-positive essential thrombocythemia (ET) patient compared to a normal control. Filled gray histograms represent the isotype control, while overlaid open black histograms show staining with the anti-Mpl antibody. (B) Total Mpl content was assessed in platelet lysates after immunoblotting with an anti-Mpl antibody (top panel) and reprobing with an anti-β_3 integrin antibody (lower panel). Results show that Mpl levels in the patient with ET (P) are similar to those in two normal controls (C1 and C2), Mpl/β3 integrin ratio in the patient was 99% of controls.

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by densitometry and expressed as percentage of the average ratio of two normal controls assayed on the same blot, was 99%, Fig. 2B. Variability in Mpl expression in normal controls (n = 5) by immunoblotting was 100 ± 5%.

MPL transcripts by real-time RT-PCR

Considering that conflicting data have been published regarding *MPL* mRNA in MPN, as both decreased (16, 20) and normal (25, 28) levels were reported, we first studied platelet *MPL* transcripts in this cohort using real-time PCR. *MPL* mRNA levels in *MPL*W515L-negative ET patients (n = 20) did not differ significantly from controls (n = 10), *MPL/GAPDH* ratio was 0.24 (0.12–0.97) vs. 0.39 (0.21–0.78), P = 0.1. No difference was found between *JAK2*V617F-positive (n = 9) and -negative (n = 11) patients, or between patients with (n = 9) and without (n = 11) treatment (data not shown). Next, we measured mRNA levels in both *MPL*W515L-positive patients, in whom *MPL/GAPDH* ratio was 0.25 and 0.26, respectively, which was similar to that of *MPL*W515L-negative patients, and within the range of normal controls, Fig. 3.

STAT3 and STAT5 phosphorylation studies

We evaluated two key downstream TPO/Mpl molecules, STAT3 and STAT5, looking for their constitutive activation and their sensitivity to TPO-induced phosphorylation. Platelet samples from the *MPL*W515L-positive ET patient displayed no constitutive activation as measured by specific antibody detection in Western blot. Moreover, when stimulated with growing concentrations of TPO, *MPL*W515L platelets showed similar STAT3 and STAT5 phosphorylation sensitivity than normal controls, Fig. 4.

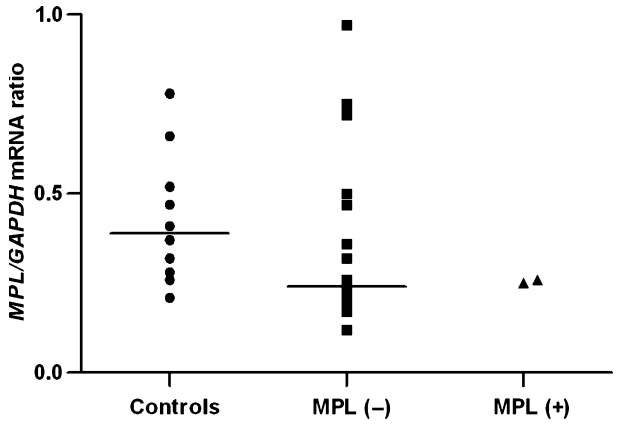

Figure 3 *MPL* transcript levels in patients positive and negative for *MPL*W515L. Total platelet RNA was prepared and reverse transcribed, and *MPL* mRNA was measured by real-time PCR after normalization with *GAPDH*. *MPL* transcript levels in both *MPL*W515L-positive patients (▲) were similar to those in *MPL*W515L-negative essential thrombocythemia (ET) patients (■), and within the range of normal controls (●). *MPL* mRNA levels in patients did not differ significantly from controls, *MPL/GAPDH* ratio was 0.24 (0.12–0.97) vs. 0.39 (0.21–0.78), P = 0.1.

Thrombopoietin levels

Plasma TPO level in the *MPL*W515L-positive ET patient was 45.8 pg/mL compared to 0 (0–32.1) pg/mL in normal controls (n = 20). Levels found in the patient were within the range of our previously studied ET cohort (n = 15), 26.4 (16–152.8) pg/mL (29).

Discussion

The low frequency of *MPL* W515L, W515K and S505N mutations found in this cohort is within the range of that found in previous studies (2, 6, 8–11, 13), highlighting the fact that the underlying molecular abnormality remains unknown in a considerable proportion of patients with ET and PMF. While W515L is the most prevalent mutant allele, comprising about 60–80% of all *MPL* mutations, followed by W515K and S505N, alternative amino acid substitutions at positions 515 and 505 have been detected in rare cases, including W515A, W515R, and S505C (13, 30, 31). The allele-specific PCR assays we used were not designed to screen for these latter mutations, while melting-curve analysis allows for detection of all exon 10 mutant alleles, which might slightly increase mutation detection rate. Lineage-specific distribution was assessed in the ET patient, revealing the presence of *MPL*W515L in myeloid lineages, including platelets, granulocytes and monocytes, and in B cells. The low levels of the mutant allele found in the T-cell

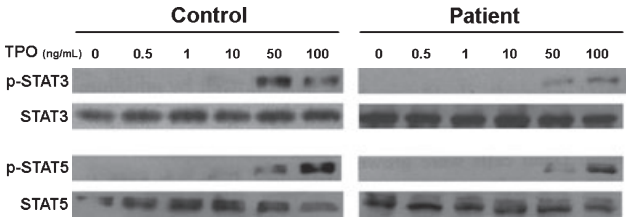

Figure 4 Phosphorylation of STAT3 and STAT5 in platelets before and after stimulation with increasing concentrations of thrombopoietin (TPO). Platelets were studied in resting conditions or after incubation with 0.5, 1, 10, 50, 100 ng/mL TPO, lysed, and subjected to SDS-PAGE and immunoblotting with anti-p-STAT3 and anti-p-STAT5 antibodies. Total STAT 3 and STAT5 loading was assessed by incubation with anti-STAT3 and anti-STAT5 antibodies. No basal STAT3 and STAT5 activation was found, and sensitivity to TPO-induced phosphorylation was not enhanced in the patient sample (right panel) compared to a healthy control (left panel).

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fraction by allele-specific PCR, but not by sequencing, could reflect T-cell involvement by the mutant clone or low levels of contamination by non-T cells. Lymphoid lineages have been shown to be involved in the *MPL*W515L/K-positive clone in a patient with unclassified MPN (32) and in some PMF patients (33), but not in others (34), indicating that at least in certain ET and PMF cases the mutation occurs at the level of a lymphomyeloid progenitor.

To explore whether differences in clinical phenotype between *MPL*W515L-positive ET and PMF could be attributed to differences in mutant allele burden, and considering that the megakaryocytic lineage is predominantly involved in *MPL*-positive disease, we studied relative amounts of *MPL*W515L in platelet-derived cDNA. While only the mutant allele was detected in the PMF patient, mutant allele burden was lower than 50% by both sequencing and real-time PCR in the ET patient, suggesting that higher levels of *MPL*W515L might favor a PMF phenotype. In this regard, Schnittger *et al.* (13) reported that 75% of patients with PMF had *MPL*W515L/K allele burden higher than 50%, compared to 27% of ET patients and, more recently, Hussein *et al.* (11) reported that fibrotic stage PMF showed higher incidence of cases with > 50% *MPL*W515L compared to both prefibrotic PMF and ET.

To determine whether *MPL*W515L leads to impaired Mpl expression, we studied Mpl at the protein and RNA level in patients positive for this mutation. Normal Mpl protein expression in platelets was demonstrated by two different techniques, including flow cytometry and Western blot, while *MPL* transcript levels were within the range of normal controls, indicating that this mutation does not interfere with normal receptor synthesis, processing and surface localization. This is consistent with the studies that show that deletion of the KWQFP motif in murine Mpl, which includes the tryptophan residue target of *MPL*W515L, does not affect its cell surface expression (3). Decreased Mpl expression represents a molecular hallmark in MPN patients, although the significance of this finding, as well as its relevance as a diagnostic tool, remains to be fully clarified. Although low Mpl levels were initially reported in a large proportion of ET patients (16, 19, 20), a more recent study showed that this abnormality is present in about half of them (18). This defect is more frequent in patients positive for *JAK2*V617F (18), suggesting a biologic relationship between Mpl expression and the underlying molecular pathogenesis. This study shows, for the first time, the presence of normal Mpl levels in an MPN patient harboring a mutation in the *MPL* gene. Therefore, although impaired Mpl expression can arise from a molecular mechanism different from *JAK2*V617F, this phenotypic abnormality seems not to be linked to *MPL*W515L. Study of a larger number of *MPL*W515L-positive patients would be required to determine the scope of this observation. In addition, the effect of different *MPL* mutations and mutant allele burden in Mpl expression would be worth of further study.

Recent data show that transgenic mice expressing reduced levels of Mpl have paradoxical thrombocytosis through decreased TPO-clearance, which results in increased stimulus for platelet production (35, 36). In this line, counterintuitively, the decrease in megakaryocyte and platelet Mpl found in MPN might in itself lead to increased platelet counts. On the contrary, the lack of Mpl down-modulation in this *MPL*W515L-positive patient indicates that this abnormality does not contribute to thrombocytosis in this case, rather suggesting that expression of mutant Mpl is required for disease pathogenesis. In this regard, studies using FDC-P1 cells show that ligand-independent activity of *MPL*W515L requires cell surface expression (5), implying that this event is required for *MPL*W515L-induced disease.

We next evaluated whether *MPL*W515L leads to constitutive phosphorylation of key molecules involved in the TPO/Mpl pathway, including STAT3 and STAT5, or whether phosphorylation of these Mpl-targets occurs at low TPO concentrations, reflecting increased sensitivity to this cytokine. Results show that although constitutive activation of TPO downstream molecules and hypersensitivity to TPO has been shown in cell lines and animal models harboring the mutant allele (2), these events could not be demonstrated in platelet samples from this patient. Discrepant results regarding this issue have been recently found in studies involving megakaryocytes. While Grimwade *et al.* (37) reported no abnormality in STAT5 phosphorylation status in megakaryocytes from patients carrying *MPL* mutations by immunohistochemistry, Gibson *et al.* (38) showed an abnormal phospho-STAT5 pattern in one *MPL*W515L-positive patient, likely reflecting STAT5 activation. In this regard, although activated STAT5 has been shown in megakaryocytes from *JAK2*V617F-positive patients (39), in a previous work, we did not find constitutive STAT5 activation in platelets from ET patients, irrespective of their *JAK2*V617F mutation status (26). Failure to detect constitutive activated STATs in this cell population may be attributed to the fact that STATs are rapidly dephoshorylated in platelets, owing to the lack of nucleus, where STATs are protected from inactivation (40). Alternatively, the potential inhibitory effect of anagrelide in TPO-signaling pathways in this patient cannot be ruled out, as interference with TPO-induced phosphorylation has been demonstrated *in vitro* (41).

Several studies have shown that hematopoietic progenitor cells from MPN patients are hypersensitive to several

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cytokines, including EPO, GM-CSF, IL-3 and IGF-I, and ET patients show enhanced megakaryocyte colony growth in response to TPO (42). Although spontaneous megakaryocyte growth has been demonstrated in *MPL*W515L-positive patients (6), dose response of cultured megakaryocytes to TPO, represented by the TPO concentration which induces half-plateau number of total clones, did not differ from normal controls (34). Likewise, in this work, we did not find increased response to TPO regarding phosphorylation of signaling substrates in the patient with ET, whose relative *MPL*W515L allele burden was $< 50\%$. It would have been interesting to assess whether enhanced phosphorylation could be demonstrated in the PMF patient, who harbored 100% mutant allele burden. Unfortunately, fresh cells were not available from this patient, who died after leukemic transformation.

In conclusion, we show that *MPL*W515L-positive platelets do not display the defect in Mpl expression characteristic of MPN, providing additional information regarding Mpl regulatory mechanisms in these disorders. Although the transforming capacity of *MPL*W515L is related to its ability to activate the receptor, in this work we did not find spontaneous phosphorylation of Mpl effectors nor enhanced response to its ligand in platelet samples. Study of additional Mpl signaling substrates would be useful to further explore this issue.

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