Deep sequencing reveals double mutations in cis of *MPL* exon 10 in myeloproliferative neoplasms

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ABSTRACT

Somatic mutations of *MPL* exon 10, mainly involving a W515 substitution, have been described in *JAK2* (V617F)-negative patients with essential thrombocythemia and primary myelofibrosis. We used direct sequencing and high-resolution melt analysis to identify mutations of *MPL* exon 10 in 570 patients with myeloproliferative neoplasms, and allele specific PCR and deep sequencing to further characterize a subset of mutated patients. Somatic mutations were detected in 33 of 221 patients (15%) with *JAK2* (V617F)-negative essential thrombocythemia or primary myelofibrosis. Only one patient with essential thrombocythemia carried both *JAK2* (V617F) and *MPL* (W515L). High-resolution melt analysis identified abnormal patterns in all the *MPL* mutated cases, while direct sequencing did not detect the mutant *MPL* in one fifth of them. In 3 cases carrying double *MPL* mutations, deep sequencing analysis showed identical load and location in *cis* of the paired lesions, indicating their simultaneous occurrence on the same chromosome.

Key words: myeloproliferative neoplasm, *MPL* exon 10, deep sequencing, mutation.

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Introduction

The *JAK2* (V617F) mutation is found in more than two-thirds of patients with a myeloproliferative neoplasm (MPN), and more specifically in about 95% of patients with polycythemia vera (PV) and 50-60% of those with essential thrombocythemia (ET) or primary myelofibrosis (PMF).[1] Activating somatic mutations of *MPL* exon 10, mainly involving a W515 substitution, have been detected in *JAK2* (V617F)-negative patients with ET and PMF.[2-11] Germline mutations of *MPL* have been found to be responsible for hereditary thrombocytosis,[12-15] and patients with the familial thrombocytosis associated with *MPL* (S505N) have been reported to have a high risk of developing splenomegaly and bone marrow fibrosis, i.e. typical myeloproliferative features.[16] In addition, a functional *MPL* polymorphism (G1238T, K39N) may be associated with a clinical phenotype of thrombocytosis in African Americans.[17]

The optimal approach to the detection of *MPL* mutations in MPN has not yet been defined. In this work we used direct sequencing and high resolution melt (HRM) analysis to identify somatic mutations of *MPL* exon 10 in 570 patients with MPN. A subset of mutated patients was further characterized using allele specific PCR and deep sequencing.

Design and Methods

We evaluated patients with myeloproliferative neoplasms followed at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo and University of Pavia, Pavia, Italy. This study was approved by the institutional ethics committee (Comitato di Bioetica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy). The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after patients provided written informed consent.

Diagnosis of PV, ET and PMF was initially made according to the criteria in use at the time of first observation, and patients were then reclassified according to the revised World Health Organization (WHO) criteria using the available data and revising the existing bone marrow biopsy.[18] The criteria of the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) were employed for the diagnosis of post-PV MF and post-ET MF.[19] Overall 570 patients with sporadic MPN were investigated for *MPL* mutations, as summarized in Table 1.

DP and AB contributed equally to this work.

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Table 1. WHO classification of the 570 cases of MPN studied and information on mutational status of *JAK2* and *MPL*.

Condition	Total N. of patients	N. of patients with JAK2 (V617F) (%)	N. of patients without JAK2 (V617F) (%)	N. of patients with somatic mutations of MPL exon 10		
				N.	% of all patients	% of patients without JAK2 (V617F)
PV*	57	56 (98%)	1 (2%)	0		
ET**	417	234 (56%)	183 (44%)	28¶	7%	15%
PMF	96	58 (60%)	38 (40%)	6	6%	16%
Total n. of patients with ET or PMF	513	292 (57%)	221 (43%)	34¶	7%	15%

**Of 57 patients with PV, 23 were in chronic phase while 34 had evolved into myelofibrosis (post-PV myelofibrosis). One of the 56 patients with mutant* JAK2 *carried a* JAK2 *exon 12 mutation. **Of the 417 patients with ET, 393 were in chronic phase while 24 had evolved into myelofibrosis (post-ET MF). ¶One of the ET patients with* MPL *mutations also carried* JAK2 *(V617F).*

Peripheral blood granulocytes and T lymphocytes were isolated as previously described.[20] DNA extraction was performed using the Puregene Blood DNA isolation kit (Qiagen, Germantown, MD, USA) according to the manufacturer's procedure. Granulocyte *JAK2* (V617F) mutation burden was assessed using a quantitative polymerase chain reaction (qPCR)-based allelic discrimination assay[21] and applying the standard curve method.

MPL mutation scanning was performed on granulocyte and T lymphocyte gDNA using both HRM analysis and direct sequencing. These methods are described in the *Online Supplementary Appendix*. Patients who were found to be *MPL* mutated at the HRM analysis but not at sequencing were further investigated using the Ipsogen MPL MutaScreen™ kit (Ipsogen, Marseille, France) for the detection of *MPL* (W515L) and (W515K) mutations. These investigations were conducted on the Rotor-Gene™ 6000 real-time analyzer. The sensitivity of this TaqMan allelic discrimination assay is approximately 1.5% mutant alleles.

For deep sequencing, PCR was performed in 50 μL containing 100 ng DNA, 1 unit of Platinum Taq DNA polymerase high fidelity together with 1x buffer and 2 mM $MgSO_4$ (Invitrogen, Carlsbad, CA, USA), 200 μM dNTPs and 200 nM of each primer (*Online Supplementary Table S2*). Cycling conditions were as follows: 95° C for 2 min (one cycle); 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec (35 cycles); 72°C for 10 min (one cycle). Aliquots of 500-1000 ng of amplified regions were purified by MinElute columns (Qiagen, Valencia, CA, USA) in order to remove the fragments less than 70 bp. Ligation of the purified samples to specific adaptors, preparation of the single-stranded libraries (ssDNA) and their amplification in emulsion were performed following the manufacturer's instructions (Roche, Basel, Switzerland). The reactions were recovered by isopropanol emulsion breaking and enriched for positive reaction beads. Each enriched sample was separately loaded onto one-sixteenth of the PicoTiterPlate (PTP) and was sequenced according to the 454 GS-FLX Titanium protocol. The resulting data were analyzed with the GS-FLX amplicon variant analyzer (AVA) software (Roche), version 2.0.01.12.

Results and Discussion

Overall 570 patients with MPN were studied for *MPL* exon 10 mutations, and results are reported in Tables 1 and 2. Based on the detection of abnormal melting curve profiles in granulocytes but not in T lymphocytes, 34 patients were found to carry a somatic mutation of *MPL* exon 10. While no *MPL* mutation was detected in 57 patients with PV, somatic mutations were detected in 34 of 513 (6.6%) of all patients with ET or PMF. The prevalence of these mutations was equal to 33 of 221 (14.9%) in patients with these conditions who were *JAK2* (V617F)-negative. One patient with ET carried both *JAK2* (V617F) and *MPL* (W515L) in circulating granulocytes.

The 34 patients with abnormal HRM profiles were studied by direct sequencing on both granulocyte and T-lymphocyte DNA. As shown in Table 2, 27 out of these 34 patients carried somatic mutations of *MPL* identified by direct sequencing. The remaining 7 patients were studied by means of the TaqMan allelic discrimination assay, and 6 of them were found to carry *MPL* (W515L). The remaining patient (MPC07_336) was scored as wild type, because neither the W515L nor W515K alleles were detected, suggesting that other lesions, different from W515L and W515K, were present at a mutant burden higher than 1% but lower than 5-10%, which are the limit of sensitivity of HRM and sequencing, respectively, as reported in the *Online Supplementary Appendix*.

In 3 patients (PV06_799, PV03_110 and MPC07_349) HRM analysis generated abnormal melting plots clearly distinguishable from patterns associated with a single mutation (Figure 1A shows 2 of the 3 patients with double *MPL* mutations in comparison with the patient PV05_558 carrying the *MPL* W515L mutation). Direct sequencing showed double mutations in these cases: W515L combined with the novel S505C (A1513T) mutation in patient PV06_799, W515L combined with the novel V501A (T1502C) mutation in patient PV03_110, and W515R combined with V501A in patient MPC07_349 (Figure 1A and Table 2).

Considering the *MPL* mutations identified in this study (Table 2), *MPL* (W515L) was the most common (67% of cases, including double mutations); *MPL* (W515K) and *MPL* (W515A) were found in 15% of cases each, and W515R in just one case (3%).

In order to investigate the 3 patients found to carry two different *MPL* mutations (Table 2), we used the GS-FLX Titanium sequencing technology. The reads generated were aligned to the reference genomic sequences, and were then grouped into 4 categories according to the possible *cis* or *trans* location of the 2 mutations: 1) Wt/Wt if no sequence variations were present; 2) Mut/Mut if the 2 mutations were detected on the same strand; 3) Wt/Mut if the mutations were present only at codon 515; 4) Mut/Wt if the sequence variation was detected only at codon 501 or 505. As shown in Figure 1B, in all cases deep sequencing clearly detected the Wt/Wt and Mut/Mut fragments (blue and green bars, respectively), while the Wt/Mut and Mut/Wt reads were present at very low or negligible percentages (orange and yellow bars, respectively) not distinguishable from the background. Thus, in these MPN patients carrying two different *MPL* lesions, both sequence variations were found to be located in *cis* on the same allele and to have the same mutation load, and no significant percentages of reads with a single mutation could be detected.

This study defines a diagnostic strategy for detecting *MPL* mutations in MPN. Our findings indicate that the

Table 2. Molecular features of the 34 patients who carried somatic mutations of *MPL* exon 10 in circulating granulocytes.*

UPN	Sex (M/F) /Age (y)	Diagnosis at the time of study	HRM analysis	*MPL* exon 10 Direct sequencing	TaqMan allelic discrimination assay for *MPL* W515K/L
PV02_66¶	F/61	ET	Mutated	Wild type	W515L
MPC07_470	M/63	ET	Mutated	Wild type	W515L
PV04_241	F/49	ET	Mutated	Wild type	W515L
PV05_613	F/35	ET	Mutated	Wild type	W515L
PV04_663	F/42	ET	Mutated	Wild type	W515L
MPC07_289	F/80	ET	Mutated	W515K	ND
MPC07_527	F/77	ET	Mutated	W515L	ND
MPC07_335	F/50	ET	Mutated	W515L	ND
PV04_584	F/65	ET	Mutated	W515L	ND
MPC08_394	F/72	ET	Mutated	W515L	ND
MPC07_10	F/58	ET	Mutated	W515L	ND
PV04_466	F/76	ET	Mutated	W515L	ND
PV06_799	F/44	ET	Mutated	S505C+W515L	ND
PV05_558	F/45	ET	Mutated	W515L	ND
PV02_541	F/50	ET	Mutated	W515A	ND
MPC07_349	F/60	ET	Mutated	V501A+W515R	ND
PV02_617	F/36	ET	Mutated	W515K	ND
PV06_878	F/47	ET	Mutated	W515A	ND
PV02_52	F/53	ET	Mutated	W515L	ND
PV03_587	F/75	ET	Mutated	W515A	ND
PV06_155	F/46	ET	Mutated	W515K	ND
PV02_100	M/68	ET	Mutated	W515K	ND
PV04_494	M/77	ET	Mutated	W515A	ND
MPC07_336	M/51	ET§	Mutated	Wild type	Negative
MPC07_247	F/76	ET§	Mutated	W515L	ND
MPC07_222	M/50	ET§	Mutated	W515A	ND
MPC07_6	M/64	ET§	Mutated	W515L	ND
PV02_113	F/66	ET§	Mutated	W515K	ND
PV05_710	M/82	PMF	Mutated	Wild type	W515L
PV03_111	F/70	PMF	Mutated	W515L	ND
MPC07_479	M/65	PMF	Mutated	W515L	ND
PV06_886	F/58	PMF	Mutated	W515L	ND
PV03_110	F/63	PMF	Mutated	V501A+W515L	ND
MPC07_305	M/84	PMF	Mutated	W515L	ND

*UPN indicates unique patient number (despite different codes, all patients came from the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy); ND, not done. *In all 34 patients, studies of DNA from circulating T lymphocytes showed wild-type* MPL *exon 10. ¶This patient was also positive for* JAK2 *(V617F) with 7.9% mutant alleles in circulating granulocytes. §Progressed to myelofibrosis.*

HRM analysis of granulocyte DNA represents an optimal approach for scanning for *MPL* exon 10 mutations in patients with MPN, confirming the recent observations by Boyd *et al.*[10] Although the most common *MPL* mutations are associated with distinct HRM profiles, a second test is required to properly identify the underlying mutation in a patient with abnormal HRM plot. Direct sequencing is clearly useful, but has a low sensitivity, and in this study it did not detect about one fourth of cases carrying *MPL* (W515L). TaqMan allele discrimination assays have a much better sensitivity but our findings indicate that they should include not only PCR for *MPL* (W515L) and *MPL* (W515K), but also a PCR for *MPL* (W515A) and possibly (W515R). The availability of a deep sequencing technology allows not only the characterization of any *MPL* mutation, but also precise assessment of the mutant allele burden as shown in Figure 1B, and might become a diagnostic procedure in the near future.

Using a sensitive allele specific PCR for *JAK2* (V617F) and the described HRM assay for *MPL* exon 10 mutations, we detected a pathogenetic clonal marker in 64% of patients with ET, post-ET MF or PMF. This figure is lower than the 71% obtained by Boyd *et al.*[10] in a study on a smaller patient population (175 patients *vs.* 513 in the current study). As both studies used the same diagnostic criteria for MPN and very similar diagnostic assays, the above difference can only reflect a different composition of the 2 study populations. Apart from *JAK2* (V617F) and

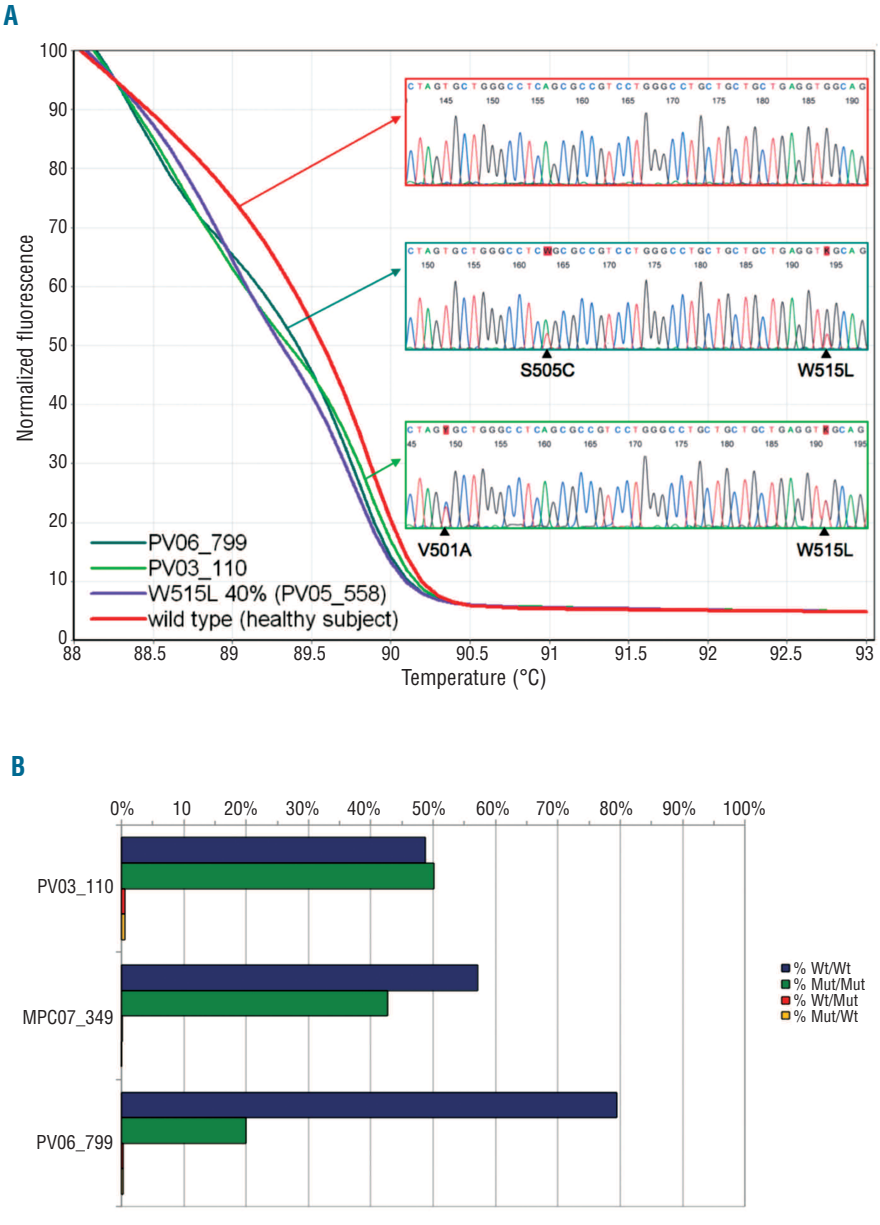

Figure 1. Mutation analysis of *MPL* in patients with MPN. (A) Abnormal HRM profiles in 2 of 3 patients carrying double mutations in *MPL* exon 10 (PV06_799 and PV03_110) compared to wild-type (healthy subject) and W515L (PV05_558) controls. These profiles were generated by the simultaneous presence of the W515L mutation in combination with the novel V501A or S505C mutations, as shown by sequencing chromatograms. (B) Deep sequencing analysis of MPN patients carrying double mutations of *MPL*. In order to investigate the 3 patients carrying two different *MPL* mutations (Table 2), we used the GS-FLX Titanium sequencing technology. In this platform individual template molecules are amplified within droplets of an emulsion, allowing the sequencing of a single DNA strand and, consequently, the characterization of the *cis* or *trans* allelic location of each set of the two *MPL* sequence variants. The large set of reads generated (PV03_110 n=3846, MPC07_349 n=2082, PV06_799 n=7070) was aligned to the reference genomic sequences, yielding a highly redundant representation of the target regions. The reads were grouped into 4 categories according to the possible *cis* or *trans* location of the 2 mutations: 1) Wt/Wt if no sequence variations were present; 2) Mut/Mut if the 2 mutations were detected on the same strand; 3) Wt/Mut if the mutations were present only at codon 515; 4) Mut/Wt if the sequence variation was detected only at codon 501 or 505. Results are presented as % of reads over the totals, providing the mutant allele burden.

somatic mutations of *MPL* exon 10, acquired mutations in *LNK* have been found in ET,[22] but they may occur only infrequently. Our study also confirms the observation by Beer *et al.*[6] that the frequency of MPN patients carrying both *JAK2* (V617F) and *MPL* mutations is negligible, supporting the concept of different genetic backgrounds underlying the occurrence of *JAK2* and *MPL* molecular lesions.

We identified 3 patients with double mutations in *MPL* exon 10. Two similar patients have been recently described by Boyd *et al.*[10] who genotyped hematopoietic colonies in a patient with ET carrying both *MPL* (W515L) and (S505N), and concluded that these mutations had arisen on different chromosomes. Our deep sequencing studies provided opposite results, clearly indicating that the paired mutations arose on the same chromosome (Figure 1B). The probability for a double mutation in the same gene to occur spontaneously is clearly extremely low, but also the probability for two mutations to occur by chance alone in the two alleles of the same individual is very low. This suggests that patients with *MPL* mutated MPN may have a particular genetic predisposition to acquiring *MPL* exon 10 mutations, which is not related to *JAK2* nor to *MPL* itself.[23] Interestingly, a double L611V/V617F in *cis* mutation of *JAK2* has been described in patients with polycythemia vera.[24]

Authorship and Disclosures

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