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

Daniela Pietra,[1] Angela Brisci,[2] Elisa Rumi,[1] Sabrina Boggi,[1] Chiara Elena,[1] Alessandro Pietrelli,[3] Roberta Bordoni,[3] Maurizio Ferrari,[2,4,5] Francesco Passamonti,[1] Gianluca De Bellis,[3] Laura Cremonesi,[2] and Mario Cazzola[1]

[1]Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo & University of Pavia, Pavia; [2]San Raffaele Scientific Institute, Genomic Unit for the Diagnosis of Human Pathologies, Center for Genomics and Bioinformatics, Milan; [3]Consiglio Nazionale delle Ricerche, Istituto di Tecnologie Biomediche (CNR-ITB), Segrate; [4]Università Vita-Salute San Raffaele, Milan; and [5]Diagnostica e Ricerca San Raffaele SpA, Milan, Italy

Citation: Pietra D, Brisci A, Rumi E, Boggi S, Elena C, Pietrelli A, Bordoni R, Ferrari M, Passamonti F, De Bellis G, Cremonesi L, and Cazzola M. Deep sequencing reveals double mutations in cis of MPL exon 10 in myeloproliferative neoplasms. Haematologica 2011;96(4):607-611. doi:10.3324/haematol.2010.034793

Online Supplemental Appendix

Detection of MPL exon 10 mutations

For the HRM assay, the optimized intronic primers reported in the Supplemental Table S1 were employed. PCR was performed in 20 μL containing 100 ng DNA, 0.5 units of HotStart Taq polymerase together with 1x buffer (Qiagen, Hilden, Germany), 1.5 mM $MgCl_2$, 800 μM dNTPs, 300 nM of each primer, and 1x EvaGreen (Idaho Technologies, Salt Lake City, UT) as intercalating dye. Cycling and HRM analysis were conducted on the Rotor-Gene™ 6000 real-time analyzer, applying the following thermal protocol: 95°C for 15 min (one cycle); 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec (50 cycles); 72°C for 10 min (one cycle); melt from 85°C to 95°C rising at 0.1°C per sec. Data were analyzed using the associated Rotor-Gene™ 6000 Series software (v1.7.87). Normalization bars were between 88°C and 88.5°C for the leading range, and 92.5°C and 93°C for the tailing range, and the confidence threshold was 90%: if the HRM plot was outside the confidence range with respect to the assigned reference genotypes, the software identified the sample as a variation.

Figure S1A shows the HRM profiles of DNA samples from a healthy subject and 3 MPN patients previously genotyped through a microelectronic microchip assay (data not shown). Patient PV02_113 was homozygous for *MPL* (W515K) (TGG>AAG conversion), and her HRM curve was shifted left towards a lower temperature with respect to wild-type sequence, as expected by a homozygous variant causing a decrease in melting temperature (Tm). Patient PV04_494 was homozygous for *MPL* (W515A) (TGG>GCG conversion) alleles, and his HRM curve was shifted right towards a higher temperature, as expected by a homozygous variant causing an increase in Tm. Patient PV05_558 had about 40% *MPL* (W515L) (TGG>TTG conversion) mutant alleles; the alteration in the shape of her HRM curve was a result of destabilized heteroduplex annealing between wild-type and mutant strands, a distinctive of a heterozygous variation.

DNA samples from patients PV02_113 and PV04_494 were then used as fully mutated control samples to check the sensitivity of HRM in detecting *MPL* exon 10 mutations in a background of non-mutated alleles. Supplemental Figure S1B shows HRM profiles and sequencing chromatograms of samples containing serial dilutions of the two mutants mixed with different proportions of wild type samples. Under optimized conditions, both mutations were clearly detected with different limits of sensitivity, HRM analysis being more sensitive than sequencing, with thresholds ranging from 1% to 5% for the HRM assay and from 5% to 10% for sequencing. Moreover, distinct HRM plots were obtained depending on both mutation type and percentage of mutant alleles.

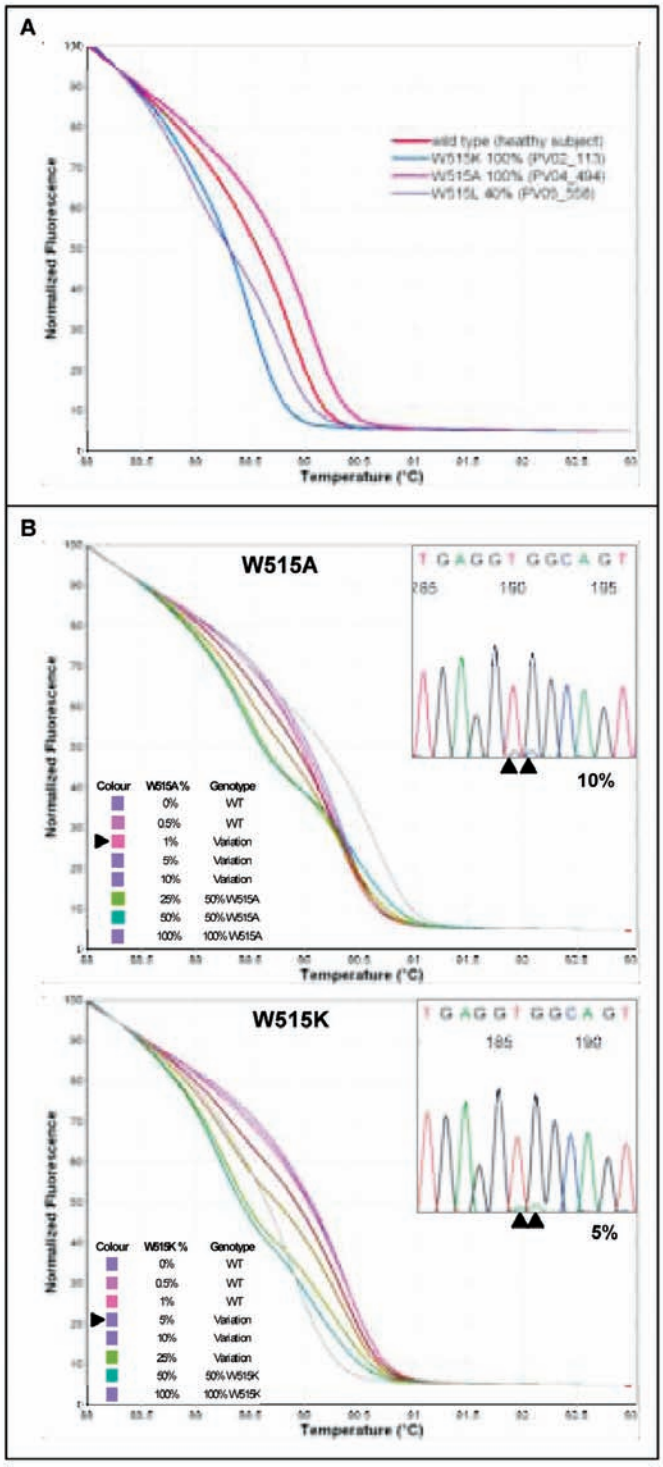
For direct sequencing, the optimized primers reported in the Supplemental Table S1 were employed. PCR was performed in 50 μL containing 100 ng DNA, 200 μM dNTPs, 1x buffer Qiagen (1.5 mM $MgCl_2$), 2.5 units of HotStarTaq polymerase with 1x buffer (Qiagen) and 400 nM of each primer. Cycling conditions were as follows: 95° C for 15 min (one cycle); 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec (38 cycles); 72°C for 10 min (one cycle). Sequencing was performed using the Big Dye Terminator v1.1 Cycle Sequencing kit and ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Online Supplementary Table S1. Primers used for *MPL* exon 10 mutation analysis.

Method	Primer	Amplicon (bp)
HRM	F 5'-GCCGAAGTCTGACCCTTTTT-3' R 5'-ACAGAGCGAACCAAGAATGCCTGTTTACA-3'	209
Sequencing	F 5'-TGACCTTGCGGGCCGAC-3' R: 5'-GATCTGGGGTCACAGAGCGA-3'	329

Online Supplementary Table S2. Primers used for 454 Roche GS-FLX Titanium sequencing.

Primer	Amplicon (bp)
F 5'-GTCTCCTAGCCTGGATCTCCT-3'	200
R 5'-GATCTGGGGTCACAGAGCGA-3'	



Online Supplementary Figure S1. HRM analysis of *MPL* exon 10. (A) HRM profiles of DNA samples from a healthy subject and three MPN patients with the most common mutations, i.e., *MPL* (W515L), *MPL* (W515K) and *MPL* (W515A). (B) HRM profiles and sequencing chromatograms of samples containing serial dilutions of the two mutants - *MPL* (W515A) and *MPL* (W515K) - mixed with different proportions of wild type samples. These samples were used to determine the sensitivity of HRM and sequencing. The lowest proportion detected is indicated by black arrowheads.