A Sensitive Detection Method for MPLW515L or MPLW515K Mutation in Chronic Myeloproliferative Disorders with Locked Nucleic Acid-Modified Probes and Real-Time Polymerase Chain Reaction

Alessandro Pancrazzi,*† Paola Guglielmelli,*† Vanessa Ponziani,**† Gaetano Bergamaschi,† Alberto Bosi,*† Giovanni Barosi,‡ and Alessandro M. Vannucchi*†

Acquired mutations in the juxtamembrane region of MPL (W515K or W515L), the receptor for thrombopoietin, have been described in patients with primary myelofibrosis or essential thrombocythemia, which are chronic myeloproliferative disorders. We have developed a real-time polymerase chain reaction assay for the detection and quantification of MPL mutations that is based on locked nucleic acid fluorescent probes. Mutational analysis was performed using DNA from granulocytes. Reference curves were obtained using cloned fragments of MPL containing either the wild-type or mutated sequence; the predicted sensitivity level was at least 0.1% mutant allele in a wild-type or mutated sequence; the predicted sensitivity level was at least 0.1% mutant allele in a wild-type or mutated sequence. None of the 60 control subjects harbored a novel, recurrent, molecular abnormality. In the test patients, 19 (8.7%) harbored the MPLW515L mutation, 10 (52.6%) with the W515L allele. In one case, both the W515L and W515K alleles were detected by real-time polymerase chain reaction. By comparing results obtained with conventional sequencing, no erroneous genotype attribution using real-time polymerase chain reaction was found, whereas one patient considered wild type according to sequence analysis actually harbored a low W515L allele burden. This is a simple, sensitive, and cost-effective procedure for large-scale screening of the MPLW515L/K mutation in patients suspected to have a myeloproliferative disorder. It can also provide a quantitative estimate of mutant allele burden that might be useful for both patient prognosis and monitoring response to therapy. (J Mol Diagn 2008, 10:435–441; DOI: 10.2353/jmoldx.2008.080015)
transmembrane-cytoplasmic hinge region that prevents spontaneous activation of the receptor.\(^2\) In fact, the tryptophan to leucine (W=L) substitution confers factor-independent growth to Ba/F3 cells that is associated with constitutive activation of downstream signaling pathways.\(^1\) Expression of \(MPL\) in a murine bone marrow transplantation model resulted in an acute myeloproliferative disorder phenotype that recapitulated several aspects of human myelofibrosis.\(^1\) In humans, the \(MPL\) mutation occurs in multipotent hematopoietic stem cells, myeloid cells, purified B and T lymphocytes, and natural killer cells.\(^2\)–\(^4\)

The \(MPL\) mutation has been detected in 5 to 7% of patients with PMF and in 1% of those with ET.\(^5\)–\(^8\) It has not been described in PV or myelodysplastic syndromes, including refractory anemia with ringed sideroblasts and thrombocytosis,\(^9\) or in acute myeloid leukemia.\(^10\) The coexistence of the \(MPL\) mutation has also been reported.\(^5\)–\(^8\) In a study of 217 patients with PMF, we found that this mutation was associated with more severe anemia and greater transfusion support.\(^11\) Therefore, a search for \(MPL\) mutations in either PMF or ET may have both diagnostic and prognostic implications; indeed, the discovery of any of these molecular abnormalities establishes the presence of a clonal myeloproliferative disorder phenotype that recapitulated several aspects of human myelofibrosis.\(^1\) The recent proposal for revision to the World Health Organization, representing a major diagnostic criterion in the human myelofibrosis.\(^1\) In humans, the \(MPL\) maps to human chromosome 1p33–34.\(^1\) GenBank sequence AL139289 was used for the design of primers and probes.\(^2\)

Previously published assay methods for \(MPL\) mutations have been represented by direct sequencing and melting curve analysis; these methods have a low sensitivity, unable to detect fewer than 10% and 3% mutant cells in a wild-type background, respectively, and are not suitable for high-throughput screening. Therefore, the aim of this study was to develop a novel real-time polymerase chain reaction (PCR) assay that could be used in a large series of patients and would be sensitive enough to detect a low \(MPL\) mutant allele burden. The latter point might be relevant to novel drugs that target members of the constitutively activated JAK/STAT pathway, as they are expected to enter the therapeutic scenario soon.\(^2\)

This novel real-time PCR assay was validated by retrospectively analyzing a population of 217 patients with myelofibrosis who had been previously genotyped using direct sequencing.\(^2\)

Materials and Methods

Patients

The characteristics of 217 patients with myelofibrosis analyzed in this study were previously described in detail.\(^2\) 150 patients had PMF, 30 had prefibrotic myelofibrosis,\(^1\) and 37 had postpolycythemic/post-thrombocythemic myelofibrosis. Sixty healthy blood donors were used as controls. Furthermore, we evaluated 50 patients with PV who harbor the JAK2V617F mutation and were diagnosed according to the proposal for revision to the World Health Organization criteria.\(^2\) All patients and controls gave their consent for sample donation and informed consent for the study.

Sample Preparation

Peripheral blood granulocytes were separated by differential centrifugation over a Ficoll-Ipaque gradient. Briefly, 20 ml of whole blood were collected into EDTA-containing polypropylene tubes and processed within 4 hours. Blood was diluted 1:1 with Ca\(^2+\)/Mg\(^2+\)-free phosphate-buffered saline, carefully layered over 15 ml of Ficoll-Ipaque in a 50-ml tube, and centrifuged at 800 \(\times\) g at room temperature for 20 minutes. Both the upper fraction and the Ficoll layer were carefully removed, while the lower fraction was collected and transferred to a fresh tube. Lysis of red blood cells was performed by the addition of a 10X volume of 1X BD PharmLyse solution (Becton Dickinson BD, San Jose, CA), centrifugation of the tube after a 15-minute incubation at room temperature, and removal of the supernatant. This step was repeated twice. After two washes in Ca\(^2+\)/Mg\(^2+\)-free phosphate-buffered saline, the dry granulocyte pellet was stored at \(-20°C\) until processed.

Table 1. Sequences of Primers and Probes Used for Real-Time PCR Assay of the \(MPL\) or \(W_{515}\)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Real-time PCR primers/probes</th>
<th>Primer/probe Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MPL) forward primer</td>
<td>5’-agcgttgacgctctggtgac-3’</td>
<td></td>
</tr>
<tr>
<td>(MPL) reverse primer</td>
<td>5’-acgccggctctcgct22-3’</td>
<td></td>
</tr>
<tr>
<td>(MPL) wild-type probe</td>
<td>5’-ctgctgagg T15gcag +T+Tc-3’</td>
<td></td>
</tr>
<tr>
<td>(MPL) 515W-L probe</td>
<td>5’-cgctgagg T15gcag +T+Tc-3’</td>
<td></td>
</tr>
<tr>
<td>(MPL) 515W-K probe</td>
<td>5’-tgctgagg A+A+Ag cagggccc-3’</td>
<td></td>
</tr>
</tbody>
</table>

LNA bases are indicated by a capital letter with a plus sign before it. Probes were labeled with 5-carboxylfluorescein at their 5’ termini while Black Hole Quencher-1 was attached to the 3’ termini.

The underlined nucleotides correspond to the 515 codon; the wild-type sequence is TGG, the \(W_{515}\) sequence is TTG, and the \(W_{515}\) sequence is AAG.
with the advantage that amplification of mutant and wild-type alleles has the same efficiencies, while specificities are obtained with different probes that were modified according to the LNA chemistry (Sigma-Proligo, Paris, France). Real-time PCR was performed using both the ABI Prism 7000 platform and the StepOne real-time PCR system (Applied Biosystems, Foster City, CA) with similar results. Forty nanograms of granulocyte DNA were used in each real-time PCR assay. Three different real-time reactions were set up in triplicate (one each for W515L, W515K, and wild-type control) for each DNA sample. A 20-μl reaction contained 1X TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol/L each primer, and 200 nmol/L each LNA-modified probe. Control wells without template (NTC) were included in each assay. Amplification and detection were performed under the following conditions: initial hold at 50°C for 2 minutes, hold at 95°C for 10 minutes followed by 55 cycles at 95°C for 15 seconds and 66°C or 62°C for 1 minute for the case of MPL unmutated and W515L probe or W515K probe, respectively. The fluorescent signal intensities were recorded and analyzed during PCR amplification using the SDS software (Applied Biosystems). The mean ΔC_{T} of triplicate determinations (C_{m}^{MPLW515L/K} - C_{m}^{MPL-wild-type}) was calculated, and the percentage of mutant alleles in the sample was obtained by comparison with a reference curve of serial dilutions of mutant plasmid mixtures in wild-type plasmid DNA. Both positive and negative controls were included in each assay.

**Preparation of Cloned MPL Fragments**

To prepare a reference curve for the quantification of the mutant alleles, a 277-bp fragment of MPL from healthy subjects (wild-type sequence) or from patients harboring either the W515L or W515K mutation was subcloned into the pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). The sequences of the forward and reverse primers for PCR were 5’-TGCGCCGAAAGTCTGCCCTTT-3’ and 5’-AGAGGTGACGTGAGCGAAGTGGCAGAAG-3’, respectively. Recombinant plasmids were isolated and bi-directional sequencing was performed using the pCR2.1 TOPO vector (Invitrogen, Groningen, The Netherlands). A 20-μl reaction contained 1X TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol/L each primer, and 200 nmol/L each LNA-modified probe. Control wells without template (NTC) were included in each assay. Amplification and detection were performed under the following conditions: initial hold at 50°C for 2 minutes, hold at 95°C for 10 minutes followed by 55 cycles at 95°C for 15 seconds and 66°C or 62°C for 1 minute for the case of MPL unmutated and W515L probe or W515K probe, respectively. The fluorescent signal intensities were recorded and analyzed during PCR amplification using the SDS software (Applied Biosystems). The mean ΔC_{T} of triplicate determinations (C_{m}^{MPLW515L/K} - C_{m}^{MPL-wild-type}) was calculated, and the percentage of mutant alleles in the sample was obtained by comparison with a reference curve of serial dilutions of mutant plasmid mixtures in wild-type plasmid DNA. Both positive and negative controls were included in each assay.

**Comparison of Conventional Sequencing and Real-Time PCR Assay**

DNA samples from 217 patients with myelofibrosis were genotyped for the MPLW515L/K mutation using conventional sequencing. MPL mutation sequencing was performed by amplifying a 248-bp region of MPL exon 10 using the following primers: forward, 5’-TAGGCTGTGATCTCCTTGGTG-3’ and reverse, 5’-AGAGGTGACGTGAGCGAAGTGGCAGAAG-3’. PCR products were subjected to bidirectional sequencing analysis using an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) as described.

**Results**

In preliminary experiments we attempted to conduct a real-time PCR assay for the MPLW515L/K allele using conventional 5’-5-carboxyfluorescein- and 3’-Black Hole Quencher-1-labeled probes, with lengths varying from 16 to 28 bp; these probes were differentially spaced along the mutation site. However, we failed to obtain satisfactory results due to a high background generated by nonspecific binding of the probes for mutant alleles to the wild-type sequence (not shown in detail). Therefore, we subsequently explored the use of LNA-modified probes because of their anticipated greater specificity of hybridization and thermal stability. Both the primers and LNA probes, listed in Table 1, were chosen for their optimal performance among other sequences and were used in all of the experiments described herein. These probes were derived from a 28-bp probe originally designed for conventional PCR, progressively shortened by the introduction of LNA residues at various positions so that the final calculated T_{m,s} of the individual probes were similar. The T_{m,s} of the probes selected from a panel of five different sets that were experimentally tested in real-time PCR assays were 73°C, 74°C, and 72°C for the wild-type, W515L, and W515K probes, respectively. The probe corresponding to codon 515 had two LNA nucleotides for the mutant sequences and one LNA base in the wild-type sequence (underlined in Table 1). In our original assay design, we performed single real-time PCRs for each of these probes, all labeled with 5-carboxyfluorescein; however, subsequent experiments showed that comparable results could be obtained by using the 5-carboxyfluorescein-labeled wild-type probe with one of the 5-VIC-labeled mutant probes in the same reaction tube. Results presented in this manuscript were obtained using the single-probe assay.

Standard curves were constructed for the wild-type and mutated sequences using serial dilutions of cloned MPL fragments in the range of 1 to 10^{6} copies; amplification plots and corresponding regression lines are presented in Figure 1. As suggested by the slope of the curves, the efficiency of real-time PCR was almost absolute. Next, we performed progressive dilutions of each mutant plasmid allele, from 0.1% to 100%, in a solution of wild-type plasmid allele as in nonhomogeneous DNA samples used for diagnostic purposes. Figure 2 shows assay performance for the W515L and W515K alleles, which reproducibly allowed for the detection of at least 0.1% mutated allele in a wild-type background. This sensitivity level could be attained using 40 ng of genomic DNA from diagnostic samples, based on a 3.7 pg of DNA content per human haploid cell. To ascertain whether a similar level of sensitivity could be obtained in clinical
samples, dilution experiments were also performed using 100% of mutated W515K or W515L patient DNA in an wild-type control DNA sample. As shown in Figure 3, the assay performance in genomic DNA samples was super-imposable on the standard reference curve generated with plasmid DNA, suggesting that that 0.1% mutant allele could be reproducibly detected in granulocyte DNA samples.

The intra-assay variation coefficient was calculated using DNA samples from 10 control subjects and four patients with either the W515L or W515K mutation and assayed in triplicate in the same plate. The variation in the calculated percentage of mutant allele was less than 2% and 1% for the W515L and W515K allele, respectively, while no control well was positive. The interassay variation test was performed using DNA samples from two patients, each with a 100% W515L or W515K allele, either undiluted or diluted in normal DNA to 10 and 50% of the mutant allele. These reference samples were analyzed in 10 different runs over a 3-month period, and the variation in the percentage of mutant allele was less than 2.5% for both mutated alleles. Therefore, this high reproducibility and the comparable performance of the reference curves obtained with plasmid or patient DNA (Figure 3) suggests that the assay does not require a standard curve for each run, but that the percentage of mutated allele in clinical samples may be obtained directly by the \( \Delta C_T \) calculation.

Under these experimental conditions, we evaluated sixty healthy blood donors, and none was found to have MPL mutant cells. Similarly, none of the 50 patients with PV had the MPLW515 mutation. We analyzed a population of 217 patients with myelofibrosis, both primary and secondary forms, who were previously genotyped using conventional bi-directional sequencing; this series has been already reported. According to sequencing results, nine patients presented with the W515L allele, eight patients had the W5151K allele, and one patient had both the W515L and W515K mutant alleles. Unfortunately, we were unable to collect fresh cells from this subject to evaluate whether these two mutations were either harbored simultaneously by a single cell or involved two different clonogenic progenitors. All patient results were confirmed using the novel real-time PCR assay. However, an additional patient who had been considered wild type using the sequencing approach was actually found to harbor low levels of the W515L allele (1.5%) by real-time PCR, and was accordingly rediagnosed as an MPL mutant. The mean calculated burden of MPL mutant alleles using real-time PCR was 51 ± 15% (range, 1.5–100%), not statistically different from that calculated using peak area integration of the chromatogram (55 ± 20%; range, 19–100%).

**Discussion**

Currently, genotyping of MPL mutations is performed using conventional bidirectional sequencing, a method that has a detection limit of 10 to 15%, is time consuming, and is relatively expensive; thus, it is not suitable for wide population screening. A melting curve technique, with a sensitivity of 3 to 5%, has also been described. We have developed a novel method for the detection and quantification of MPLW515L/K mutations that is based on LNA probes for real-time PCR. LNA probes have increased thermal stability and hybridization specificity.
due to their higher \( T_m \), making them especially suitable for allelic discrimination. Indeed, LNA chemistry was key to the development of our assay in which a number of conventional TaqMan probes tested initially displayed very poor specificity. Using the optimized conditions described here, we were able to attain a sensitivity level of at least 0.1% mutant allele in a wild-type background, a value that is at least 100-fold higher than conventional sequencing. This high sensitivity of the assay is of particular relevance in the setting of myeloproliferative disorders in which only a proportion of hematopoietic clones and their differentiated progeny might harbor \( MPL \) mutations. It is also conceivable that the true incidence of \( MPL \) mutations in myeloproliferative disorders has been underestimated in previous studies where direct sequencing or melting curves were used for genotyping. As a matter of fact, one patient in this series who had been considered wild-type \( MPL \) using sequencing harbored a low W515L allele burden by real-time PCR. Furthermore, in a large series of patients with ET, we found that the frequency of \( MPL \) mutations using the real-time PCR assay was sevenfold higher than originally reported.

The studies described herein have been conducted on purified granulocytes; due to the retrospective design of the study, we do not have stored samples of whole blood or bone marrow to perform comparative analyses among these different cellular sources. However, even in the case of \( JAK2\) V617F genotyping, it is still unknown whether fewer homogeneous cellular samples compared with granulocytes might be equally informative.

In summary, we have developed a sensitive and convenient method for the detection and quantification of \( MPL \) mutations at \( MPL \) codon 515 based on real-time PCR in the presence of LNA-modified probes. This methodology is expected to facilitate the screening of \( MPL \) muta-
tions in large patient series, helping to molecularly classify patients with ET or PMF and possibly provide a prognosis. In addition, this approach might be useful for measuring changes in mutant allele burdens in patients who will receive, in the near future, novel targeted drugs against MPL mutant cells and/or components of the JAK/STAT pathway, as well as in assisting disease monitoring after hematopoietic stem cell transplantation in myelofibrosis as described for the JAK2V617F mutation. However, assessment of the role of the MPLW515 mutant allele burden quantification as a biomarker of disease or minimal residual disease will require prospective studies.

Acknowledgments

We thank all of our colleagues who referred patients for genotyping, and we thank patients and healthy donors for their contributions to the study.

References


28. Lasho TL, Pardanani A, McCutie RF, Mesa RA, Levine RL, Gilliland DG, Tefferi A: Concurrent MPL515 and JAK2V617F mutations in