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A sensitive detection method for MPLW515L or MPLW515K mutation in myeloproliferative disorders

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ABSTRACT

Myeloproliferative Leukemia Virus (MPL) appearsdistinctly associated withpolycythemia vera (PV), essential thrombocythemia (ET) and Primary myelofibrosis(PMF). Currently available methods for the detection of MPL mutations are considered amongst diagnostic laboratories as expensive and hard to follow. Here we propose asimple and robust in-house multiplex semi-nested polymerase chain reaction (PCR)assay for the detection of both the MPLW515L and MPLW515K mutations loads for as lowas 0.5% for both mutations in a single reaction. The assay is based on specific primersdesigned for each mutation and is recommended for the screening of MPLW515L/Kmutations in patients suspected to have a myeloproliferative disorder. Unlike thecommercially available kit and the various available detection methods for thesemutations, this assay is both cost-effective and sensitive. The simplicity of this assaycan ensure its wide use in almost all clinical diagnostic laboratories.

Keywords: MPL; MPL W515L; MPL W515K; primer; PCR.

INTRODUCTION

Myeloproliferative Neoplasms (MPN) are clonal hematopoietic stem cell disorders leading to the abnormal accumulation of one or more types of blood cell in the bone marrow[1].In early 2005, a number of novel mutations were identified and described in BCR-ABL-negative MPNs, out of which mutations in Janus kinase 2 (*JAK2*) and Myeloproliferative Leukemia Virus (*MPL*) genes appeared distinctly associated with PV, ET and PMF with corresponding mutational frequencies of ~ 99, 55 and 65% for *JAK2* and 0, 3 and 10% for *MPL* mutations [1]. Recently, somatic frameshift mutations in exon 9 of the calreticulin (*CALR*) gene were also identified in ET and PMF patients [2,3].

MPL is located on chromosome 1p34 and encodes for the receptor forthrombopoietin,the key growth and survival factor for megakaryocytes [1]. *MPLW515L* was first described in2006 amongst*JAK2 V617F*-negative PMF patients and is the most frequent MPN-associated*MPL* mutation, resulting from a G to T transition atnucleotide 1544 on exon 10, causing a tryptophan to leucinesubstitution at codon 515 [1]. Somatic *MPL* mutations are rare,stem cell-derived events involving both myeloid andlymphoid progenitorsand limited to MPN patients[4,5].

An assay kit for semi-quantification of common *MPL* mutations is commercially available by Qiagen, namely*ipsogen*® MPL W515L/K Muta*Screen*,as well as several other methods that have been proposed for the detection of these mutations, such as real-time PCR [6], high resolution melting [7] and deep sequencing [8], have been proposed for the detection of these mutations. However, these methods have been characterized as being either, expensive, time-consuming and/or hard to pursue.Following our recent publication of in-house PCR assays for the detection of *JAK2 V617F* and *CALR* mutations [9], we hereinpropose a PCR assay for the detection of *MPL* mutations, with a sensitivity of 0.5% for both W515L and W515K. This assay constitutesa simple, sensitive, and cost-effective procedure for the screening of *MPL*W515L/K mutations in patients suspected to have a myeloproliferative disorder,based on specific primers designed for each mutation.

MATERIALS AND METHODS

DNA samples of patients referred to our laboratory for investigation of possible myeloproliferative disorder over the last 6 years were used to validate the proposed method. These patients were previously screened and confirmed to carry either of the *MPL*W515L/K mutations using the Qiagen*ipsogen*® MPL W515L/K Muta*Screen* detection kit (Qiagen, Cat. Number:676413).

PCR reactions were set up and standardized for the two mutations, usingcommon forward and reverse primers. Primers were designed using the online Primer3 primer design tool (http://primer3.ut.ee/). A mismatch base for W515L was also introduced to increase the specificity (Table 1). For the detection of the W515L mutation, 50ng of genomic DNA were amplified in 25µl reaction volume with 0.03pmol/µl forward, 0.3pmol/µl reverse primer and 0.9pmol/µl of mutation specific forward primer. For the detection of the W515K mutation, 50ng of genomic DNA were amplified in 25µl reaction volume with 0.3pmol/µl forward, 0.06pmol/µl reverse primer and 0.9pmol/µl of mutation specific forward primer. Primer sequences are listed in Table 1. Both reactions were carried out usingHotStartTaq™ at 95˚C for 10 min followed by 40 cycles at 95˚C for 30 sec, annealing at 54˚C for 30 sec and 72˚C for 30 sec, finishing with 72˚C for 10 min.

Table 1. Forward, reverse and mutation specific primer sequences used in the study

W515L	F: GCCGAAGTCTGACCCTTTTT	209bp
	R: ACAGAGCGAACCAAGAATGCCTGTTTACA	
	SSPF: GGCCTGCTGCTGCTGAA**G**Tt	124bp
W515K	F: GCCGAAGTCTGACCCTTTTT	209bp
	R: ACAGAGCGAACCAAGAATGCCTGTTTACA	
	SSPR: TGTAGTGTGCAGGAAACTGCtt	125bp
Bold letter = mismatched base, small letters = mutation sites.		

RESULTS AND DISCUSSION

Samples referred to our laboratory for MPN testing that were confirmed to carry either the W515L or W515K mutation by the Qiagen*ipsogen*® MPL W515L/K Muta*Screen* detection kit. All samples were analyzed for the presence of *JAK2 V617F*, *MPL* codon 515 and *CALR* exon 9 indel mutations. A total of 8 samples were found to carryan *MPL* codon 515 mutation (7 W515L and 1 W515K).Using the proposed PCR method, each of the DNA samples were found to carry the mutation as originally detectedby the Qiagen*ipsogen*® MPL W515L/K Muta*Screen* detection kit. For validation purposes, DNA samples from healthy individuals, negative for any of the *MPL* mutations, were also used (data not shown).

Whilst testing the sensitivity of these assays, the mutation load of samples was adjusted to 10%, 5%, 2.5%, 1% and 0.5% with WT DNA. The proposed assay demonstrates a sensitivity of 0.5% for both *W515L* and *W515K* mutations (Figure 1).For the reliability of daily diagnosis tests we propose to use 1% as cut off.

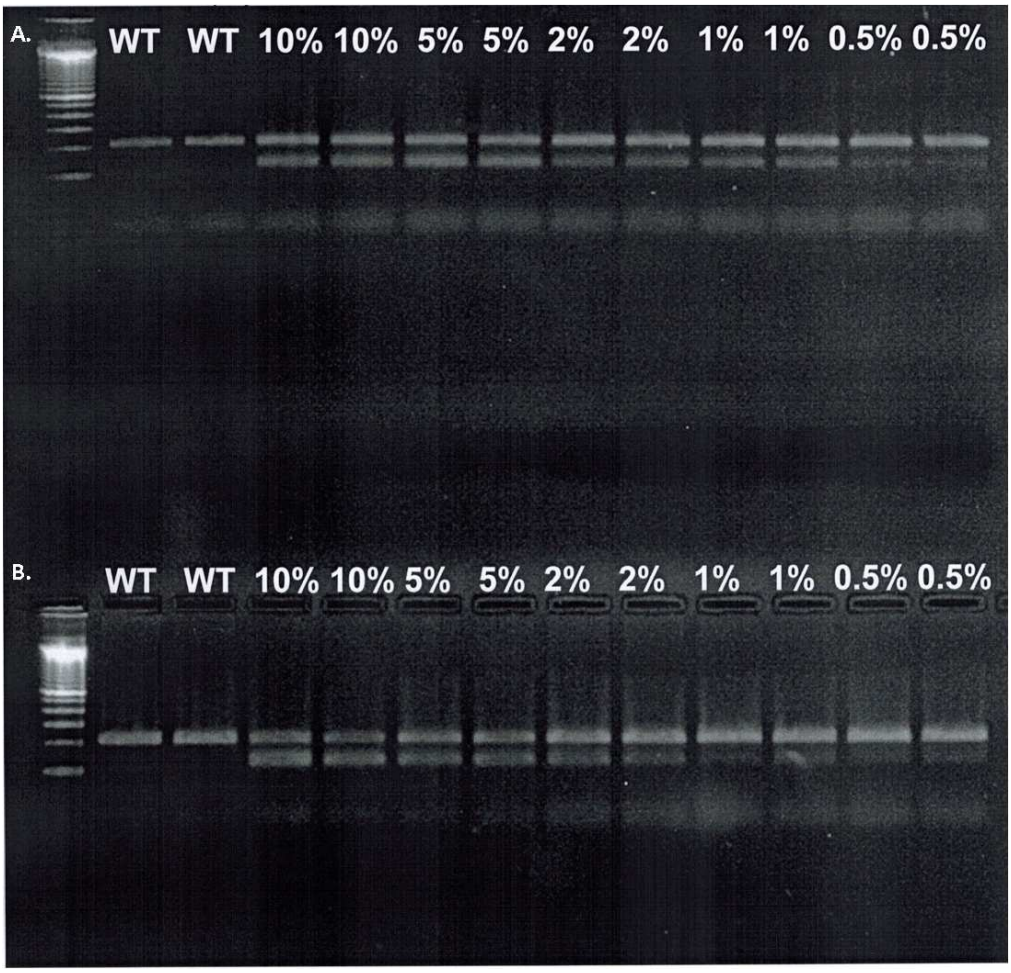

Figure 1. Specificity and sensitivity analysis for MPL W515L and W515K mutations. Mutation load of as low as 0.5% is detectable for both W515L (A) and W515K (B) mutations. Each assay was tested against a WT control and samples of different mutation loads, adjusted using WT DNA. All reactions were performed in duplicate

CONCLUSION

In summary, we are proposing an alternative PCR method for the detection of both W515L and W515K mutations in the *MPL* gene. We assessed whether this assay can be used to detect these two mutations using DNA samples frompatients previously confirmed to carry these mutations using a commercially available kitand then we standardized and validated this assay for use in a molecular diagnostic laboratory. All mutated samples used to test this method resultedin confirming the initial findings. The sensitivity of the technique was also assessed in order to examine its usefulness in detecting minimal loads of the mutations in a wild type background. The multiplex semi-nestedPCR assay could detect the mutations at a load of 0.5% for both W515L and W515Kin a single reaction, making it a cost-effective and sensitive technique that consistently achieved detection of both *MPL* mutations in samples referred to our laboratory.

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