CORRESPONDENCE

Allele Specific PCR: A Cost Effective Screening Method for MPL Mutations in Myeloproliferative Neoplasms

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Dear editor,

Philadelphia negative myeloproliferative neoplasm primarily includes Polycythemia Vera, Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) [1]. Majority of the ET and PMF patients have mutations in *JAK2* (*JAK2* V617F) or *CALR* genes. Mutations mapping to the juxtamembrane region of the thrombopoietin receptor *MPL* (exon 10) have been described in 5–10% of PMF patients and 1% of ET patients, who were negative for *JAK2* and *CALR* mutations [2]. These *MPL* mutations are gain of function mutations leading to receptor activation in the absence of thrombopoietin binding with constitutive activation of the JAK-STAT signaling. Spontaneous activation of this receptor leads to uncontrolled overactivity resulting in myeloproliferative neoplasms [3].

The *MPL* gene located on chromosome 1p34 has 12 exons coding for the thrombopoietin receptor. Four recurrent [c.1544G > T (W515L), c.1543_1544TG > AA (W515 K), c.1543_1544TG > GC (W515A) and c.1514G > A (S505 N)] and two rare [c.1544G > C (W515S) and c.1543T > A or c.1543T > C (W515R)] *MPL* mutations have been reported in ET and PMF patients [2, 4]. While Sanger sequencing (*MPL* exon 10) has the advantage of screening all these six mutations, it is limited

by its analytical sensitivity. Mutations occurring at allelic levels below 15% may not be detected by routine Sanger sequencing [5].

We screened a total of 80 DNA samples from patients diagnosed with ET or PMF, based on peripheral blood and bone marrow findings, in the Department of Haematology, Christian Medical College, Vellore between Jan 2010 and July 2017. All these samples were negative for *JAK2* V617F and *CALR* mutations. DNA extraction from peripheral blood leukocytes was done using a commercial kit (Gentra Puregene Blood kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Screening for *MPL* mutations was done by both bidirectional Sanger sequencing and Allele specific PCR.

As c.1544G > T, c.1543_1544TG > AA, c.1543_1544TG > GC and c.1514G > A mutations account for 98% of *MPL* mutations, we aimed to design an assay capable of detecting each of these mutations with high sensitivity and specificity [5]. We devised an Allele specific oligonucleotide PCR (ASO-PCR) based approach, using previously published protocols with specific modifications, for screening the four common *MPL* mutations [5]. While Furtado et al. performed gene scan analysis with capillary electrophoresis using labeled primers, we used an ASO-PCR based approach which is a cost effective alternative to gene scan analysis without compromising the sensitivity. Primers targeting the wild type and mutant sequences were used in this assay and the specificity of the primers was based on a 3' mismatch in the primer sequence [5]. The PCR reaction was carried out in two tubes simultaneously for each sample. The first tube had primers targeting c.1544G > T and c.1514G > A mutations. The second tube had primers targeting c.1543_1544TG > AA and c.1543_1544TG > GC mutations. The specificity of the primers was confirmed by known positive controls

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which were characterized by sequencing. The PCR amplicons are electrophoresed in an agarose gel along with a 100 base pair ladder and the interpretation of the results was based on the difference in product size.

MPL mutation was identified in 12 out of 80 patients (15%) using either of the two methods. Sanger sequencing and allele specific PCR identified the mutations in 10 and 12 patients respectively. We did not identify c.1514G > A (S505 N), c.1543_1544TG > GC (W515A) or other rare mutations in our patient cohort. Sanger sequencing failed to detect the nucleotide change in 2 patients with W515L mutation showing its limitation as a screening tool for *MPL* mutations. A typical example is shown in Fig. 1. Panels a and c show the electropherograms (forward sequences) from the same patient at presentation and after 1 year respectively where the initial screening result was equivocal. The corresponding reverse sequences are shown in b and d. Allele specific PCR with the same samples (Panel g lanes 1 and 2) showed a clear positivity at both the time points.

Although direct sequencing has an advantage of screening all possible mutations in exon 10 of *MPL* it is limited by its low sensitivity which is a major concern while dealing with somatic mutations. We performed serial dilution assay to establish the Limit of Detection (LOD) of ASO-PCR and its superiority to Sanger sequencing in terms of assay sensitivity. ASO-PCR showed a clear

positivity for *MPL* mutation when the mutant allelic burden was as low as 1.56% (Fig. 2). On the other hand Sanger sequencing produced false negative results when the allelic burden falls below 10%. Although mutant allele concentrations below 1.5% can be picked up by a repeat PCR with an increased number of amplification cycles in the ASO-PCR program (supplement table 1), the clinical significance of such low level mutant allele burden is questionable at screening level. The specificity of the assay was also validated by using 40 known control samples [including healthy controls, known CML patients with *BCR-ABL1* translocation, diagnosed MPN patients with other MPN related (*JAK2/CALR*) mutations and MPN patients with *MPL* mutations other than the one targeted by the specific ASO primer pair]. Other methods such as real time quantitative PCR, high-resolution melting and pyrosequencing have been described in the literature for the detection of *MPL* mutations [6, 7]. Although they have a similar or even better sensitivity (ranging from 0.1 to 3%) to ASO-PCR, they are limited by various factors like availability and cost issues.

ASO-PCR is a cost effective and sensitive screening platform for *MPL* mutations that can be used by most laboratories. PCR Mastermix (12.5 µl × 2 per sample), primers (1.5 µl each × 2 per sample) and an electrophoresis setup are sufficient to perform the assay while sequencing demands all the above along with sequencing

Fig. 1 Representative sequencing Electropherograms and electrophoresis gel pictures of *MPL* mutation screening by Sanger sequencing and ASO-PCR respectively. Forward **a** and reverse **c** sequences of the patient's *MPL* exon 10 sequence showing equivocal results. Forward **b** and reverse **d** sequences from the same

patient tested after 1 year showing clear positivity for *MPL* W515L (c.1544G > T) mutation. **e, f** Normal Electropherograms for comparison. **g** ASO-PCR showed clear positivity at both the time points (lane 1—at presentation and lane 2—after 1 year) highlighting its superiority as a screening method for *MPL* mutations

Fig. 2 Electrophoresis image showing the serial dilution assay to assess the sensitivity and lowest limit of detection (LOD) for MPL ASO-PCR. DNA samples from a known patient with MPLW515L mutation and a normal control were mixed in different proportions to get a gradient of mutant allelic burden as shown in the image. Mutation was detectable even when the mutant allelic burden was as low as of 1.56% (arrow in the image) while the result was equivocal when the mutant allele burden was 0.78%

and clean up procedure reagents and a capillary electrophoresis unit which is not available in most of the labs in our country. In our experience we find the cost of sequencing to be approximately twice as high as ASO-PCR for MPL mutations. Further, the turnaround time for the assay is also less (4 h from PCR reaction setup till interpretation of the result) when compared to Sanger sequencing, which is generally a two day procedure in our lab. However ASO-PCR has its own limitations as well. ASO-PCR cannot be used to quantitate mutant allele burdens and presence of mutations other than the four mutations cannot be ruled out. This can be possibly overcome by using a third optional tube with primers specific to the two rare mutations [c.1544G > C (W515S) and c.1543T > A or c.1543T > C (W515R)]. Laboratories can also use a combined approach where the ASO-PCR negative patients are screened subsequently by Sanger

sequencing for the rare MPL exon 10 mutations. In our study, sequencing did not yield any additional mutation other than the four common mutations tested by allele specific PCR while the high sensitivity of allele specific PCR over Sanger sequencing enabled us to pick up two additional positive cases.

Compliance with Ethical Standards

Conflict of interest The authors have no competing interest.

Ethical Approval This is a retrospective analysis using DNA extracted from EDTA blood sample that has been collected for routine molecular diagnostic procedures (JAK2, CALR) in patients with myeloproliferative neoplasms in our institution. No additional sample was collected from any patient for this study. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments.

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