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Brief communication

Novel multiplex bead-based assay with LNA-modified probes for detection of *MPL* exon 10 mutations

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ABSTRACT

MPL exon 10 mutations were the second class of mutations shown to be associated with the pathogenesis of some Philadelphia chromosome – negative myeloproliferative neoplasms (MPNs). Recently, their identification gained wide recognition in the diagnostic work-up for suspected cases of JAK2 V617F negative MPNs. Various molecular approaches have been applied, yet universally accepted method is still lacking. We aimed at development and validation of a novel bead-based liquid assay using Locked nucleic acids (LNA)-modified oligonucleotide probes for multiplexed detection of the following *MPL* mutations: W515L/K/A/R. Testing on both artificial plasmid constructs and on clinical samples revealed that the method was comparable in terms of specificity to direct sequencing and had a much higher sensitivity of 1% mutant alleles. This method could be successfully implemented in the diagnostic work-up for MPNs. Furthermore, this system allows further multiplexing for single-tube identification of different mutations associated with MPNs.

1. Introduction

After the identification of *JAK2* V617F mutations in a vast majority of cases with Philadelphia chromosome – negative myeloproliferative neoplasms (MPNs) [1] a number of other mutations that play an important role in the pathogenesis of MPNs have been described [2]. Among them MPL exon 10 mutations were shown in cases with *JAK2* V617F negative primary myelofibrosis (PMF) and essential thrombocythemia (ET) leading to constitutive activation of the JAK-STAT signaling [3,4]. Therefore, currently their identification in clinical samples is being more widely recognized as a part of the routine diagnostic work-up for MPNs [5]. Several molecular methods were reported for the identification of MPL exon 10 mutations such as allele specific polymerase chain reaction (AS-PCR) [6], direct sequencing [4], real time PCR (RT-PCR) [7] and high resolution melting (HRM) curve analysis [5]. Direct sequencing is still the gold standard in the identification of mutations. However, it remains labour intensive and has low sensitivity (~20% mutant allele burden). In contrast AS-PCR and RT-PCR assays for MPL mutations are more sensitive – around 1% mutant allele but require separate reaction for each mutation which is usually impractical. HRM provides platform for a single tube detections of numerous mutations with a sensitivity of about 4%. In order to address the requirements of the clinical practice for a sensitive multiplexed and easily applicable molecular method for detection of *MPL* exon 10 mutations we developed an accurate, sensitive and mid-throughput bead-based liquid assay [8] for detection of the W515A, W515K, W515L and W515R mutations. Furthermore, in order to enhance probe sensitivity we used Locked Nucleic Acids (LNA)-modified oligonucleotide probes [9], which might ease the future multiplexing of the assay.

2. Materials and methods

2.1. Patient samples

A total of 25 peripheral blood samples of consecutive patients with known Philadelphia chromosome-negative MPNs were collected between January 2010 and December 2010. Patients were classified according to the WHO criteria as follows: PV ($n = 12$), ET ($n = 5$), PMF ($n = 8$). The study was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all patients.

2.2. DNA extraction

All samples were collected using sodium citrate-containing blood sampling tubes (BD Biosciences) and stored at room temperature for no more than 4 h before processing. Genomic DNA was extracted from whole blood using a iPrep (Invitrogen)

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automated system. Genomic DNA samples were stored at −20 °C before further analyses.

2.3. MPL exon 10 sequencing

A fragment of *MPL* exon 10 was amplified following primers: 5′-TAGCCTGGATCTCCTTGGTG (forward) and 5′-AGAGGTGACGTGCAGGAAGT (reverse). Amplification was performed using: 100 ng genomic DNA; 1.5 U Taq polymerase (Invitrogen); 3 mM $MgCl_2$; 0.2 mM dNTP mixture; and 10 pmol of each primer in 25 μl reaction. The amplification conditions were as follows: 95 °C for 5 min; 35 cycles – 95 °C for 30 s, 62 °C for 40 s, 72 °C – 2 min and final extension at 72 °C for10 min. PCR products thus generated were purified by Exo-SAP (Applied Biosystems) and sequenced directly. The sequencing reaction was conducted in a 10-μL final volume using 2 μL of the purified PCR product, 3.2 pmol of one the PCR primers and 2 μL of Big Dye terminator cycle-sequencing kit v3.1 (Applied Biosystems). The sequencing program was a 25-cycle PCR program (denaturation at 96 °C for 10 s; annealing at 50 °C for 5 s and elongation at 60 °C for 4 min). The sequence detection was conducted using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

2.4. Bead-based assay

The fragment encompassing the *MPL* exon 10 was amplified from either genomic or plasmid DNA samples using 5′-botinylated forward primer. The same primers and PCR conditions as described above for the sequencing analysis were applied. Genotyping was performed by direct hybridization with 5 LNA-modified oligonucleotide probes, specific for the wild type the mutant alleles (designed and synthesized by Exiqon, Denmark): 5′-GAAACTGCCACCTCAGCA (MPL-wt); 5′-GAAACTGCGCCCTCAGCA (MPL-W515A); 5′-GAAACTGCTTCCTCAGCAG (MPL-W515 K); 5′-GAAACTGCAACCTCAGCAG (MPL-W515L) and 5′-GAAACTGCCTCCTCAGCAG (MPL-W515R). All probes were synthesized with 5′-amino group and 20 bases oligonucleotide spacers to allow covalent attachment to carboxylated microbeads (Luminex Corp). Immobilization of the amine modified probe to carboxylated surface of the beads was completed using a standard carbodiimine-coupling procedure. Mixtures of 5 sets of coupled microspheres were prepared by combining equal volumes of each set of beads. Approximately, 160 microspheres of each set/μl were used for analysis of one DNA sample. 5 μl of PCR product; 20 μl Hybridization buffer (Wakunaga Pharmaceuticals); 3 μl Bead mixture and 2 μl SAPE (Wakunaga Pharmaceuticals, Hiroshima, Japan) were combined in a well of a Thermowell 96-well plate and hybridization was performed at 70 °C for 30 min in a thermocycler. After incubation, 75 μl Washing buffer (Wakunaga Pharmaceuticals) were added to each reaction and the plate was centrifuged at 4000 rpm for 2 min. The supernatant was removed and the microspheres were resuspended in 75 μl Washing buffer (Wakunaga Pharmaceuticals). The samples were analyzed on a LuminexIS200 machine (Luminex Corp.). A minimum of 100 events per bead region of interest was collected. For each set of reactions a background control of a sample containing only the respective sets of beads was included. The background mean fluorescence intensity (MFI) values were subtracted from the MFI values for each sample and the resulting values were used for the calculation of the relative fluorescence indices as follows: Index(mutant allele 1) = [MFI(mutant allele 1)/[MFI(mutant allele 1) + MFI(mutant allele 2) + MFI(mutant allele 3) + MFI(mutant allele 4) + MFI(wild type allele)].

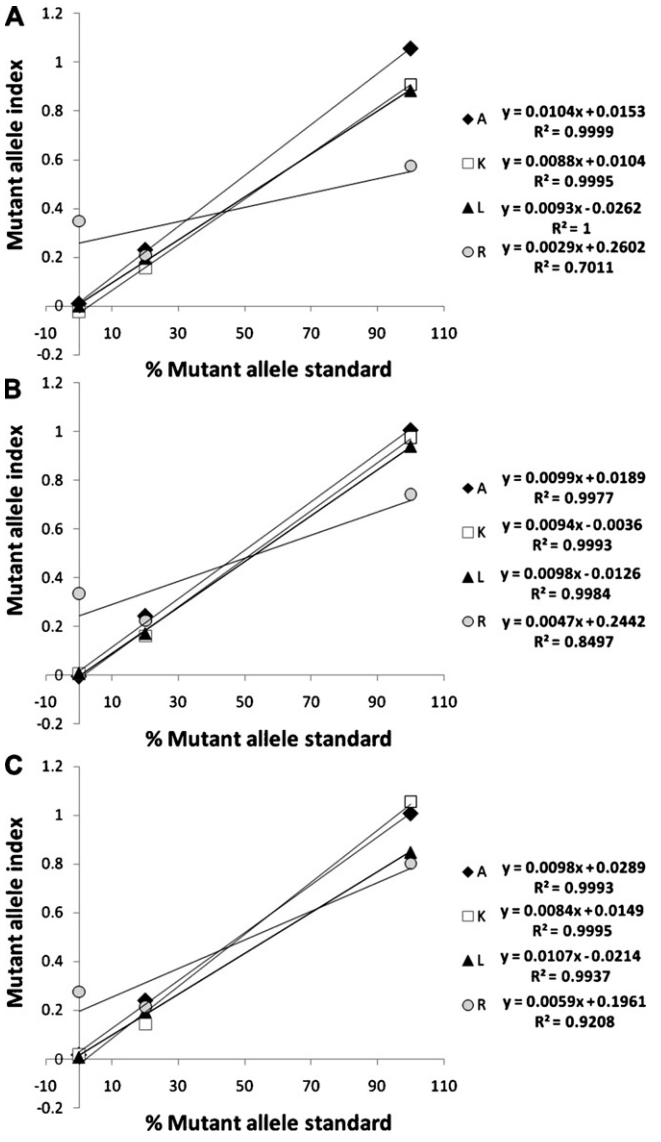

Fig. 1. Standard curves for each mutant for determination of the optimal hybridization temperature for multiplex assay. At (A) 66 °C; (B) 68 °C; (C) 70 °C.

3. Results

3.1. Determination of the hybridization conditions

In order to determine the optimal hybridization conditions we prepared dilutions of the 100% mutant plasmid with 100% wild type plasmid to generate reference samples with each allele at levels of 100, 20, 0%. For the 20% standard, a mix of equal quantities of plasmid for all mutants and the wild type was used. As the Tm for all the probes was estimated to 72 °C hybridization reactions for all the standards were set up at 66 °C, 68 °C and 70 °C. Mutant allele index for each sample was calculated and standard curves were generated for each mutant at every hybridization temperature. R^2 value for each curve was calculated showing that the highest R^2 values were obtained for the 70 °C level (Fig. 1). As high hybridization temperature can increase specificity while reducing the intensity of the fluorescent signal we calculated the delta MFI value for each allele versus every other. As it is generally accepted that this delta MFI should be above 1000 we confirmed that this condition is met for the 70 °C hybridization temperature (Fig. 2). This temperature was used for all further analyses.

3.2. Determination of the index threshold value and assay sensitivity

To determine the mutant allele index threshold value we performed the assay in triplicate with 100% standard for each allele (wild type and mutants) the maximum index for each mutant from the samples where it was not present was calculated for each run of the assay. Then the mean of the maximal values was calculated and the threshold value was defined as mean + standard deviation. The values were as follows: 0.072, 0.086, 0.038 and 0.244 for W515A, W515K, W515L and W515R, respectively (Fig. 3). The highest value was determined for the W515R, which would be expected based on the higher T_m value of 72 °C of the probe for that mutation. Then we aimed at determination of the sensitivity of the assay. To do this we tested plasmid samples with wild type only and 1%, 2.5%, 5%, 12.5% and 25% of each mutant plasmid on wild type background. Sensitivity was defined as the sample with the lowest mutant content which yielded mutant allele index above the threshold value. The sensitivity did not differ between mutants and was 1.0% for all the mutant alleles (Table 1).

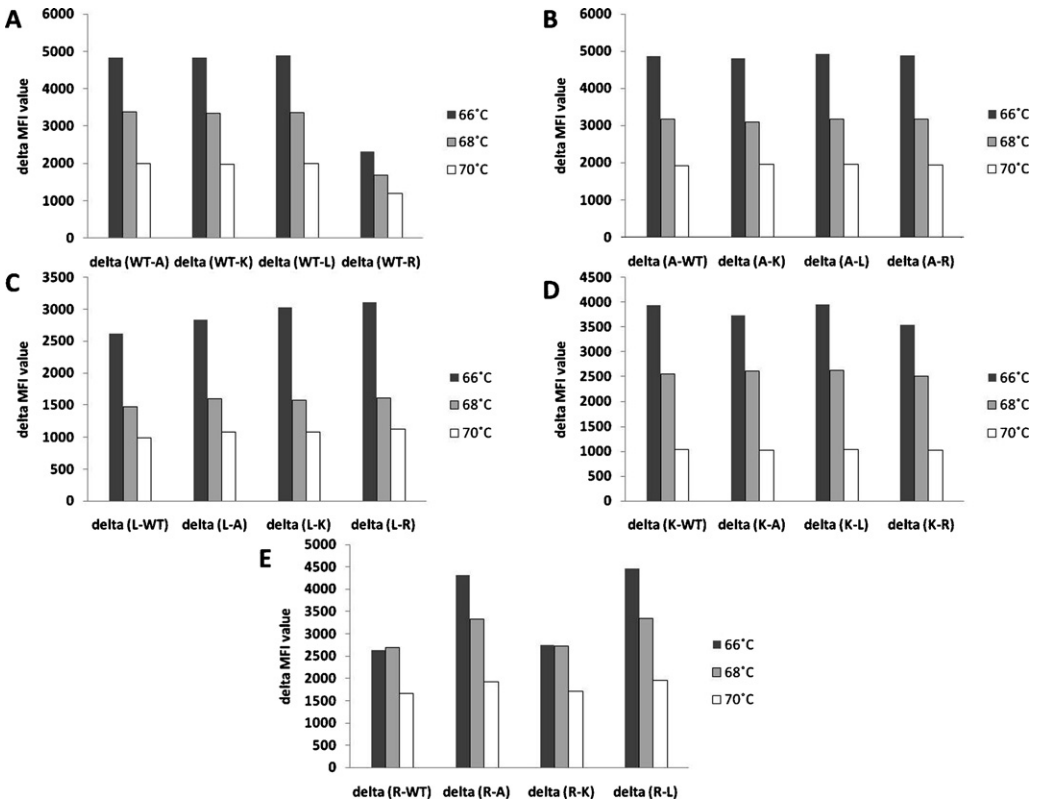

Fig. 2. Delta MFI values obtained at different hybridization temperatures for all the alleles. (A) Wild type; (B) W515A; (C) W515L; (D) W515K; (E) W515R.

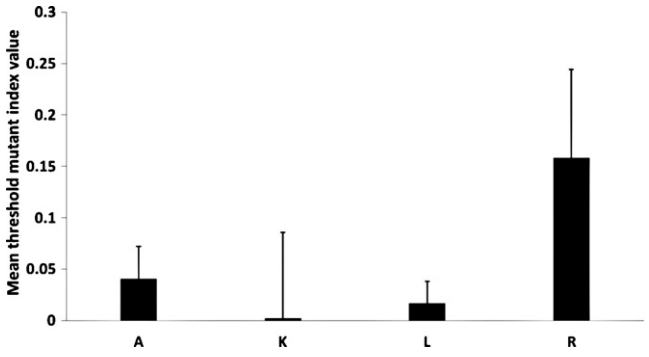

Fig. 3. Determination of the threshold value for each mutant allele index. Error bars represent the standard deviations.

3.3. Comparison of the bead-based assay with direct sequencing using patients' DNA

We further tested the performance of our bead-based assay on a set of genomic DNA samples from 25 patients with known MPNs. Only one sample showed a mutant allele index higher than the above-mentioned threshold levels. The value was 0.391 for W515R mutant and this sample was considered positive for the mutation. Indeed, we confirmed the presence of the mutation by direct sequencing. The patient had been diagnosed with JAK2 V617F negative PMF. All other samples were assessed *MPL* mutation negative on both direct sequencing and bead-based assay, which again validated our assay in clinical settings.

Table 1
Determination of the sensitivity of the bead-based assay. Values of the mutant allele index above the determined threshold values are bolded.

% Mutant allele standard	W515A	W515K	W515L	W515R
0	0.005	0.011	0.008	0.231
1	**0.088**	**0.100**	**0.094**	**0.253**
2.5	**0.145**	**0.200**	**0.160**	**0.247**
5	**0.300**	**0.240**	**0.163**	**0.264**
12.5	**0.334**	**0.444**	**0.200**	**0.373**
25	**0.488**	**0.545**	**0.366**	**0.429**

4. Discussion

Recently, the presence of *MPL* W515 mutations were shown to be associated with *JAK2* V617F negative cases of MPNs. Moreover, routine testing for those mutations has been implicated in the routine diagnostic work-up for those disorders. Several methods have already been reported for MPL mutations detection, including direct genomic DNA-sequencing [4], allele-specific polymerase chain reaction (AS-PCR) [6], real-time PCR [7,10,11] and high resolution melting (HRM) [5] curve analysis. Direct sequencing is the least sensitive method, whereas real time PCR and AS-PCR revealed sensitivity between 0.1% and 1.0% mutant allele on a wild type background. Here we reported the development of a novel liquid bead-based assay with similar sensitivity of 1.0% for all four W515 mutant variants assayed. This high sensitivity was achieved by using Locked Nucleic Acids (LNA)-modified oligonucleotide probes [5]. Indeed, LNA-modified probes have already been reported for detection of MPL mutations by real time PCR. Furthermore, Paradis et al. [12] recently reported a bead-based assay for detection of the most frequent mutation associated with MPNs (*JAK2* V617F) and recognized the main advantage of the assay – the open platform for multiplexing. Yet, here we demonstrated for the first time that such multiplexing is possible for the clinically relevant MPN mutations. Furthermore, our assay is quantitative but this has lit-

tle practical implication because MPL mutant allele burdens are generally not considered of clinical relevance [13]. In our opinion further improvement of sensitivity of the bead-based assay below 1% mutant allele would be possible through mutant enrichment by initial COLD-PCR amplification similar to the approach reported for KRAS mutations detection [14].

Finally, the bead-based assay for *MPL* W515 mutation is a rapid method as the duration of the entire procedure from DNA extraction to final data acquisition is less than 5 h. The method is less labor intensive and is cost-effective. It is quantitative and robust and LNA-modified probes lead to the clinically relevant sensitivity of 1% mutant allele. It allows also a mid-throughput analysis in 96- or 386-well plates. However, the most important advantage of the method remains the possibility to further extend the multiplexing for single-tube detection of various mutations associated with MPNs.

Conflict of interest

All authors have no conflict of interest to declare.

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Contributions. M.I.I. and V.S.S. contributed equally to this work they provided the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for important intellectual content, and final approval of the version to be submitted; E.A.H. supplied the acquisition of data; E.J.N. supplied the design of study, provided the revised the article critically for important intellectual content and gave final approval of the version to be submitted.

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