A new TMHA-DHPLC assay for the rapid mutation screening of JAK2 exon 14 in myeloproliferative disorders

To the Editor: V617F mutation in exon 14 of JAK2 gene has been reported in more than 90% of patients with polycythemia vera (PV) and in near 50% of patients with essential thrombocythemia (ET) or idiopathic myelofibrosis. Molecular screening for the presence of V617F is becoming part of the current diagnostic process for these disorders and thus rapid and reliable methods are required. Moreover, given the recent description of two novel nucleotide exchanges next to V617F [1,2], it would be preferable to have an assay able to detect all mutations of potential pathogenetic relevance in exon 14.

In a recent study [3], a method based on temperature modulated heteroduplex analysis (TMHA) through DHPLC is used. This method is unfortunately not sufficiently sensitive for V617F detection in a clinical setting. The poor sensitivity of TMHA-DHPLC method is related to the heterogeneous melting profile of the amplicons. The melting temperature of the region surrounding V617F mismatch is very different from that at the ends of the generated amplicons. Partially denaturing conditions of the region of interest are obtained at a very high temperature, causing destabilization and denaturation of the whole fragment (Fig. 1a).

To retain the advantages offered by the DHPLC technology, including its capability to potentially detect all exon 14 mutations, we devised a very fast and high-throughput modified TMHA-DHPLC assay adding a short stretch of nucleotides with a typical GC sequence to the forward primer (GC-clamp). Differently from the amplicon without GC-clamp, the new predicted melting curve was uniform for the entire target sequence (Fig. 1b,d). The addition of the clamp generated a region with a higher temperature compared with the target amplicon (Fig. 1d). Forward primer 5’-cgccgcgcgcgcgcccctatgtatggcagagaga-3’ and reverse primer 5’-cactgacacctagctgatcc-3’ was used and amplifications were performed under standard conditions. The chosen oven temperature was the highest at which the target sequence was predicted to be \( \approx 90\% \) double helix (Fig. 1c). As wild-type control, we used DNA from a subject lacking any exon 14 polymorphism. After standard heteroduplex creation, partially denaturating DHPLC screening was done in 3 min run. To evaluate the reliability of the GC-clamp TMHA-DHPLC in clinical practice, we tested 148 PV and 176 ET patients with the new method in parallel with allele-specific PCR followed by amplification refractory mutation system PCR (AS/ARMS-PCR). In addition, 20 V617F heterozygous positive samples from PV and ET patients were also tested using standard TMHA-DHPLC. The presence of AS/ARMS-PCR V617F positive patients was confirmed in all cases by using GC-clamp TMHA-DHPLC by the distinct appearance of abnormal chromatographic profiles (Fig. 1e). On the contrary, standard TMHA-DHPLC was unable to detect mutated allele in 20 positive controls, confirming its insensitivity. Serial dilutions of a mixture containing the amplification product of a PV patient homozygous for JAK2 V617F and a wild-type control were tested. The JAK2 V617F mutation was clearly detected in presence of 1 mutated allele every 12 normal alleles.

With this new GC-clamp, TMHA-DHPLC assay laborious sample preparation and costly labeling procedures are avoided. This method appears reliable, high-throughput, and sensitive for the detection of allelic variants involving JAK2 exon 14 in peripheral blood of patients with myeloproliferative diseases.

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References

Fig. 1. (a) Denaturing trends at different theoretical temperatures of the original amplicon containing JAK2 exon 14. The nucleotide region surrounding the V617F mutation was highlighted. Base pair position was plotted versus helical fraction ratio (0.00 value for 100% single stranded; 1.00 value for 100% double stranded). (b) Amplicon melting profile obtained under the original conditions without the inclusion of the 15-bases GC-clamp. The nucleotide region surrounding the V617F mutation was highlighted. Base pair was plotted versus the melting temperature ($T_m$, °C). The target sequence region is put in evidence. (c) Denaturing trends of the clamped amplicon containing JAK2 exon 14 at different temperatures. The nucleotide region surrounding the V617F mutation was highlighted. Base pair position was plotted versus helical fraction ratio (0.00 value for 100% single stranded; 1.00 value for 100% double stranded). (d) Amplicon melting profile with inclusion of a 15-bases GC-clamp on the 5' amplification primer. Amplicon melting profile is uniform along the target sequence. The nucleotide region surrounding the V617F mutation was highlighted. The target sequence and the clamped regions were put in evidence. Base pair was plotted versus the melting temperature ($T_m$, °C). (e) DHPLC chromatogram revealing the presence of the V617F heteroduplex (green line) and the wild-type homoduplex control (black line). Retention time (minutes) was plotted versus the absorbance intensity (mV). Run was performed at 56°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]