Detection of the JAK2 Mutation in Myeloproliferative Neoplasms by Asymmetric PCR With Unlabeled Probe and High-Resolution Melt Analysis

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Background: Several methods have been established to detect the JAK2 V617F mutation, a frequent event involved in the pathogenesis of myeloproliferative neoplasms (MPNs). High-resolution melt (HRM) analysis is a newly established technique without the requirement of any gel-based post-PCR handling. **Methods:** An asymmetric PCR with unlabeled specific probe was developed and combined to HRM analysis to screen for JAK2 V617F mutation. **Results:** Heterozygous mutation was easily distinguished from homozygous JAK2 for the obvious shape change. Homozygous JAK2 mutant can be also well separated from wild-type JAK2 in the presence of internal temperature calibrators. The easily recognizable and maximal

sensitivity of HRM analysis was 5% for the detection of JAK2 V617F mutation, higher than 25% of direct sequencing. In the test of blind screening of 223 samples (111 Ph⁺ MPNs, 60 Ph⁺ chronic myeloid leukemia, and 52 acute myeloid leukemia), JAK2 V617F mutations were found in 78 (70%) patients with MPNs, but in none with chronic and acute myeloid leukemia. HRM analysis of all cases was fully concordant with the results of PCR-RFLP and direct sequencing. **Conclusions:** The HRM method with unlabeled probe could be used as convenient, sensitive and reliable diagnostic test for detection of JAK2 V617F mutation. *J. Clin. Lab. Anal.* 25:300–304, 2011.

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Key words: JAK2 V617F; asymmetric PCR; unlabeled probe; high-resolution melting analysis; myeloproliferative neoplasm

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of diseases characterized by increased hematopoiesis leading to elevated numbers of non-lymphoid cells and/or platelets in the peripheral blood. Recently, a novel somatic point mutation (c.1849G>T, GTC→TTC) in JAK2 gene, which results in substitution of phenylalanine for valine (V617F), has been identified in MPNs (1–4). Because of high incidence of JAK2 V617F mutation in MPNs, detection of the JAK2 mutation has been introduced in the diagnostic criteria for MPNs by the World Health Organization as an important parameter (5).

Although several methods have been established to detect JAK2 V617F mutation, each of these methods

has its own merits and limitations, including sensitivity, ease of use, and instrumentation constraints (1–10). High-resolution melting (HRM) curve analysis is a newly established technique without the requirement of

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any gel-based post-PCR handling (11). In the presence of a saturating double stranded DNA (dsDNA) binding dye, LCGreen™, HRM analysis has shown high sensitivity and specificity for mutation scanning and single-nucleotide polymorphism (SNP) genotyping. We previously reported the validation of HRM analysis with short amplicon using LightScanner platform as a detection strategy for JAK2 V617F mutation in a diagnostic setting (12).

Mutation detection by amplicon melting depends on the specificity of the PCR primers. At least in clinical assays, it is commonly believed that the extra specificity of a probe is necessary (13). To provide probe specificity without the cost of a fluorescently labeled oligonucleotide, we investigated the feasibility of asymmetric PCR with unlabeled oligonucleotide probe and combined to HRM analysis by LightScanner™ platform in the detection of JAK2 V617F mutation.

MATERIAL AND METHODS

Subjects and DNA Extraction

Two hundred and twenty-three patients were enrolled and bone marrow (BM) samples were collected between August 2004 and October 2010. The samples included 37 cases of polycythemia vera (PV), 58 cases of essential thrombocythemia (ET), 16 cases of primary myelofibrosis (PMF), 60 cases of Ph+ chronic myeloid leukemia (CML), and 52 cases of acute myeloid leukemia (AML). All BM samples were obtained with informed consent. BM mononuclear cells were separated by density-gradient centrifugation using Ficoll. Subsequently, the DNAs were extracted using the Genomic DNA Purification Kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions.

Asymmetric PCR Conditions

Probe and primer design was performed with LightScanner primer design software v1.0 (Idaho Technology, Salt Lake City, Utah). Melting temperatures of the probe were chosen between 65 and 70°C to avoid interference with the extension and the maximum probe length was set on 35 bp. Moreover, extra care needs to be exercised in designing PCR reactions to avoid primer dimers and nonspecific amplification in HRM analysis. The sequences of primers and probe are as follows: 5'-AAGCAGCAAGTATGATGAGC-3' (forward primer), 5'-AGAAAGGCATTAGAA AGCCTGTA-3' (reverse primer), and 5'-ATGGAGTATGTTTCTGTGGAGACG-3' (probe). The unlabeled probe was blocked at its 3' end with a C6 amino modifier (Invitrogen, Shanghai, China). The temperature of melting (T_m) of PCR product (112-bp) was ~81.37°C. Two internal temperature

controls were introduced to calibrate the deviation of T_m of JAK2 product. The sequences of controls were as follows: 5'-ATCGTGATTTCTATAGTTATCTAAGTAGTTGGCATTAAATAATTT CATT-3' (low-temperature sequence, T_m ~69.6°C) and 5'-GCG-GTCAGTCGG CCTAGCGGTAGCCAGCTGCGGC-ACTGCGTGACGCTCAG-3' (high-temperature sequence, T_m ~88.9°C) (14). All synthesized oligonucleotides were all of standard molecular biology quality (Invitrogen, China).

PCR was performed in 25- μ l volumes in the presence of 1 $\times$ LCGreen Plus (Idaho Technology Inc.), 1 $\times$ PCR buffer (Invitrogen, Merelbeke, Belgium), 2.5 mmol/l magnesium chloride ($MgCl_2$), 0.2 mmol/l of each dNTP, 0.16 μ mol/l of forward primer, 0.8 μ mol/l of reverse primer, 0.8 μ mol/l of probe, 1 U Taq polymerase (MBI Fermentas, Canada), and 50 ng genomic DNA. PCR reactions were performed on 7300 Thermal Cycler (PerkinElmer Inc., Ramsey, MN). The temperature cycling protocol consists of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and an extension at 72°C during 30 sec. This PCR reaction is followed by another denaturation step at 94°C for 30 sec, and then cooling down to 25°C to facilitate the heteroduplex formation.

HRM Analysis

Ninety-six-well plate was transferred to the LightScanner™ instrument (Idaho Technology Inc.) after PCR amplification. Plate was heated in the LightScanner from 55 to 95°C with a ramp rate of 0.10°C/sec. During the course of temperature rising, the fluorescence signal is collected. The fluorescence intensity decreases as the dsDNA melts and the dye is released. The specific sequence of the dsDNA determines the melting behavior. The T_m s of internal temperature controls and JAK2 amplicon may be approximated by taking the derivative of the melting curve, which is analyzed by the LightScanner software package with CALL-IT® software (Idaho Technology Inc.). Melting profiles of JAK2 amplicon were calibrated by internal oligonucleotide controls, and then normalized, temperature shifted, grouped, and displayed as fluorescence vs. temperature plots or subtractive difference plots ($-df/dt$ vs. T).

Detection of JAK2 V617F Mutation by RFLP and Sequencing

PCR-restriction fragment length polymorphism (RFLP) analysis was performed to detect the JAK2 V612F mutation according to the previously described procedure (4). Subsequently, the PCR products were sequenced directly (Shanghai GeneCore BioTechnologies Co., Ltd., China).

RESULTS

Utility of Internal Temperature Controls

To evaluate the utility of internal temperature controls in this methodology, we amplified three samples with homozygous mutant (T/T), heterozygous mutant (G/T), and wild-type DNA (G/G) in triplicate. Figure 1 displayed a complete derivative melting profile of the amplified products before (A and B) and after (C and D) calibration. Melting signatures from the low and high internal temperature calibrators are observed at ~ 70 and $\sim 89^\circ\text{C}$ flanking the JAK2 amplicon melting peaks. Heterozygous JAK2 was easily distinguished from homozygous JAK2 for the obvious shape change. Homozygous JAK2 mutant can be only well separated from wild-type JAK2 after calibration ($\Delta\text{Ct} = 0.35^\circ\text{C}$).

Sensitivity of HRM Analysis

To assess the sensitivity of this HRM assay, the wild-type DNA was mixed with that of the homozygous mutant in a series of concentrations (containing 0, 1, 5, 10, 25, 50, and 100% mutant). The sensitivity test was carried out in duplicate for ensuring the reproducibility of the HRM analysis. The JAK2 V617F mutation can be easily distinguished in the normalized melting curves and the normalized melting peaks (Fig. 2). In the normalized melting peaks, although the homozygous

mutants displayed the same shapes as wild-type JAK2, it was easily distinguished from the wild-type JAK2 for the obvious T_m shift. The HRM assay was able to distinguish 5% of mutant DNA in a background of wild-type DNA with a good reproducibility. However, the reliable sensitivity was identified at 25% of mutated JAK2 by direct sequencing because it was difficult to clearly determine the presence of mutation for the very faint peak of 10% of mutated JAK2 (Fig. 3).

Detection of JAK2 V617F Mutation in Patients

The HRM assay was performed with a blind screening of 223 samples. Out of 111 cases with Ph⁺ MPNs, 34 (92%) PV were positive for JAK2 mutant (10 homozygous and 24 heterozygous), 38 (66%) ET positive for JAK2 mutant (6 homozygous and 32 heterozygous), 6 (38%) PMF positive for JAK2 mutant (1 homozygous and 5 heterozygous). JAK2 mutant was found in none of all patients with CML and AML. The judgments were identically interpreted by two blinded technicians. The results of HRM analysis in all cases were fully concordant with those of PCR-RFLP and direct sequencing. These gave a diagnostic specificity (number of wild-type samples detected/number of wild-type samples included in CML and AML patients) of 100% (112/112) and a diagnostic sensitivity (number of mutant

Fig. 1. Derivative melting curves of homozygous and heterozygous JAK2 V617F mutation and wild-type JAK2 generated from independent PCR reactions performed in triplicate. (A) Shifted melting peaks before calibration. (B) Normalized melting curves before calibration. (C) Shifted melting peaks after calibration. (D) Normalized melting curves after calibration.

Fig. 2. Results of HRM analysis for different concentrations of JAK2 V617F mutant diluted with wild-type DNA. 1: 0 and 1% mutant; 2: 5% mutant; 3: 10% mutant; 4: 25% mutant; 5: 50% mutant; 6: 100% mutant. (A) Normalized melting curves; (B) normalized melting peaks. HRM, high-resolution melting.

samples detected/number of mutant samples included in Ph- MPNs) of 100% (78/78).

DISCUSSION

HRM with unlabeled probe in PCR solutions can be used for genotyping of SNP or mutation that has already been known (13,15,16). The probe is included in the PCR mix, but is not consumed during amplification, since it is blocked at the 3'-end (13). The saturating dye, LCGreen, is added before asymmetric PCR and genotyping is accomplished by monitoring the melting transitions of the probe-target duplexes post-PCR. Since the double stranded region can only be as long as the probe, a high sensitivity rate can be obtained.

This study described a rapid scanning of JAK2 V617F mutation by HRM analysis using the LightScanner platform and an unlabeled specific probe. Our results indicated that the heterozygous JAK2 mutation was easily identified by HRM analysis by the characteristic shape change of the melting curve. However, variations in the PCR buffer or ionic strength between samples caused by adding the sample significantly compromised the reproducibility of T_m measurements and reduced the ability to detect homozygous JAK2 mutant. The inclusion of internal temperature calibrators significantly decreased the variance of T_m caused by sample-adding associated PCR reaction system alterations and then increased the accurate differentiation of homozygous JAK2 mutant with wild-type JAK2. According to the T_m shift, it is very easy to discriminate homozygous JAK2 mutant from wild-type DNA.

Fig. 3. Sequence chromatograms of mutated DNA diluted serially into wild-type DNA. There were obvious peaks of G>T mutation in 50 and 25% JAK2 mutants. The G>T mutation was nearly undetectable in 10% mutant. No peak of G>T mutation was observed in 5% mutant.

We further evaluated the analytical sensitivity of HRM assay with unlabeled probe and determined 5% as the easily recognizable and maximal sensitivity, which was compared with the previously reported sensitivities for PCR-RFLP, pyrosequencing, dHPLC, and real-time PCR (12). This sensitivity is similar with that obtained by the HRM assay using short amplicon without probe (12), higher than 25% of direct sequencing. These results confirmed that it was very easy to identify JAK2 V617F mutation using this HRM analysis without the need of any time- and labor-consuming postamplification steps such as restriction digest, electrophoresis, amplicon clean-up, and sequencing.

The validation of HRM assay for clinical diagnostic pre-screening purpose was further performed by examining a relatively large series of samples from 143 patients with Ph⁺ MPNs, CML and AML. Two blinded technicians described the same analytical results for all samples which were also confirmed by PCR-RFLP and direct DNA sequencing. The diagnostic sensitivity, specificity, and reproducibility of HRM method were very high.

In summary, we established a sensitive, accurate, and reliable HRM assay with unlabeled specific probe, which can be used as a clinical diagnostic test for JAK2 V617F mutation.

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