



Evaluation of v617f jak2 gene mutation by high-resolution melting method in patients with erythrocytosis

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Abstract

Although several techniques have been developed for the detection of JAK2 V617F mutation, these techniques have their disadvantages. High-resolution melting (HRM) analysis is a new, post-PCR analysis. Simple and fast, this method is based on PCR melting curve techniques. This study examined the JAK2 V617F mutation by the high-resolution melting method in 20 patients with erythrocytosis, and the results were compared with those obtained from the direct sequencing method. The results showed 100% sensitivity and 100% positive predictive value for this methodology in the patient sample set tested.

INTRODUCTION

Myeloproliferative disorders are diseases of the bone marrow and blood which can strike at any age, have no known cause, and have a wide range of symptoms and outcomes (1). In some cases, the disease progresses slowly and requires little treatment; other times it develops into acute myeloid leukemia (AML). Studies have shown that 57% of patients with idiopathic myelofibrosis carried a somatic mutation in the JAK2 gene (V617F) (2). JAK2 kinase is a member of a family of tyrosine kinases involved in cytokine receptor signaling. Moreover, it is misregulated or mutated in a number of myeloproliferative diseases and cancers (3). Mutation V617F is the most clinically relevant variant and is seen in around half of myeloproliferative disorders. The variant is a known activating mutation, and activated JAK2 is sufficient to drive myeloproliferative disorders in mouse models. V617F, while most recurrent, is not the only mechanism by which JAK2 can be activated in patients (7). JAK2 is now one of the first diagnostic markers tested upon diagnosis of a myeloproliferative disorder. The JAK2 V617F mutation causes constitutive activation of the kinase with deregulated intracellular signaling that mimics continuous hematopoietic growth factor stimulation (8). This mutation results in the substitution of phenylalanine for valine (V617F), both hydrophobic nonpolar amino acids, at position 617 of the JAK2 protein within the JH2 pseudokinase domain (9). Detection of the JAK2 mutation has recently been included under the essential diagnostic criteria for polycythemia vera, thrombocythemia, and

myeloproliferative neoplasm by the World Health Organization (WHO) diagnostic criteria. Methods for the detection of JAK2V617F mutation based on the analysis of genomic DNA obtained from peripheral blood granulocytes have been devised and are already used in clinical practice (10). High-resolution melting (HRM) analysis is a new, post-PCR analysis method. Simple and fast, this method is based on PCR melting curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)-binding dyes along with next-generation real-time PCR instrumentation and analysis software. In the current study, JAK2 V617F mutation was examined using the high-resolution melting method, and the results were compared using the direct sequencing method.

MATERIALS AND METHODS

This study included 20 cases of erythrocytosis as the patients and 20 healthy blood donors as the controls. Blood sampling was performed after informed consent was obtained either at diagnosis or during the follow-up, but free from chemotherapy. Granulocytes were separated by differential centrifugation over a Ficoll-Paque gradient. Then, patient genomic DNA was extracted by a Rapid Blood Genomic DNA Extraction Kit (BioBasic, Canada). DNA was amplified using primers JAK2 exon 12 (forward: 5'-CTCCTCTTTGGAGCAATTCA-3'; reverse: 5'-GAGAAGTTGGGAGTTGCGATA-3'). PCR products were sequenced by ABI-3130 sequencer (Applied Biosystems). A 126bp amplicon was generated using primers in JAK2 exon 12 (5'-AATGGTGTTTCTGATGTACC-3') and intron

12 (5'-AGACAGTAATGAGTATCTAATGAC-3'). Each PCR contained 40 ng DNA, 2.5 mM MgCl₂, 200 μM dNTPs, 0.5 μM of each primer, 5x Hot FIREPOL Eva Green qPCR Mix (SolisbioDYNE, Estonia). Duplicate PCRs and the HRM analysis were conducted on a RotorGene 6000™ real-time analyzer. The PCR profile was an initial hold at 95 °C for 10 mins, 40 cycles at 95 °C for 15 secs, 58 °C for 30 secs, and 72 °C for 20 secs, followed by 50 °C for 30 secs and then a melt from 70 °C to 95 °C rising at 0.1 °C/second. Normalization bars were between 72-75 °C for the leading range and 92-93 °C for the tailing range.

RESULTS

Sensitivity tests were carried out in triplicate to ensure the reproducibility of the HRM analysis. The *JAK2* V617F mutation can be easily distinguished in the normalized melting peaks and normalized difference curves. In the normalized melting peaks, the homozygous samples showed sharp, symmetric melting transitions, whereas heterozygotes had a more gradual, complex transition. Homozygous mutants were identified with a T_m shift compared with the wild-type. In the normalized difference curves, the melting profile of a wild type control was chosen as the horizontal baseline, and the relative differences in the melting of all other samples were plotted relative to this baseline. The HRM assay was able to reproducibly distinguish 5% of mutant DNA in a background of wild-type DNA. HRM was successful in discriminating DNA samples with a wild type *JAK2* exon 12 genotype from those including an exon 12 mutation, and it correctly identified all positive samples in this group with no false-positive results. Direct sequencing of HRM products confirmed the presence of an exon 12 mutation in these cases when compared with the titration experiment results.

DISCUSSION

Because this methodology will be affected by the presence of inheritable polymorphisms, a candidate region containing *JAK2* exon 12 and intron 12 was selected on the basis of an absence of polymorphisms in the NCBI database (11). Subsequently, a previously unreported polymorphism within this region was identified in 85 out of the 128 tested individuals; this variation involved the presence or absence of a penta-nucleotide sequence (position 5,060,231-5,060,235) that would significantly affect any HRM profile. Accordingly, this region was excluded from the final amplicon (12). Several techniques have been developed for the detection of *JAK2* V617F mutation; however, these techniques have their disadvantages. DNA sequencing, currently considered as the gold standard for mutational analysis because of its ability to identify the specific DNA-sequence changes, is limited by its high cost

and low sensitivity (13). Pyrosequencing offers a sensitive alternative method but requires expensive equipment. The dHPLC method can detect mutations sensitively, but it also requires a considerable initial investment for equipment. ARMS, the most sensitive method, requires the manipulation of amplified PCR products. PCR-RFLP is time-consuming and has the possibility of incomplete digestion causing false-positive result. For several real-time PCR platforms, one major disadvantage is the need for expensive fluorescence-labeled probes (14-16). Mutation analysis of granulocyte DNA samples from V617F-negative erythrocytosis patients using sequencing, allele-specific PCR, and HRM allowed the assessment of the sensitivity and positive predictive value of the HRM methodology. Mutations were not detected by allele-specific PCR or sequencing in samples scored as wild type by HRM, resulting in 100% sensitivity and 100% positive predictive value for this methodology in the patient sample set tested.

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