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Detection of the *JAK2* V617F missense mutation by high resolution melting analysis and its validation

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ABSTRACT

Background: Janus kinase 2 (JAK2) is a tyrosine kinase involved in the cytokine signaling of several growth factors such as erythropoietin and thrombopoietin in normal and neoplastic cells. The G to T exchange at nucleotide 1849 in exon 14 of the *JAK2* gene leads to a substitution of valine with phenylalanine at the amino acid position 617 (V617F) of the JAK2 protein. Currently, the occurrence of the *JAK2* V617F mutation is well recognized in chronic myeloproliferative disorders (MPDs).
Methods: We identified *JAK2* V617F missense mutation in patients with MPD by high resolution melting (HRM) analysis. HRM analysis is a new gene scan tool that quickly performs the PCR and identifies sequences alterations without requiring post-PCR treatment. This study included 7 PV patients (41.1%), 6 ET patients (35.3%), and 4 myelofibrosis patients (23.5%). Additionally, our methodology was compared with amplification refractory mutation system (ARMS) assay.
Results: Up to 5% of the *JAK2* V617F mutation was successfully detected in patients with MPD using HRM analysis. Eleven out of 17 patients (64.7%) were positive for the presence of *JAK2* V617F mutation. The prevalence of mutation in the different subtypes of MPDs was 85.7% in PV (6 of 7 patients), 66.7% in ET (4 of 6) and 5.9% in myelofibrosis (1 of 4). The results proved 100% comparable to those obtained by ARMS assay.
Conclusions: The HRM analysis is a rapid and effective technique for the detection of *JAK2* V617F missense mutation.

1. Introduction

The Janus family of tyrosine kinases (JAK) and the family of signal transducers and activators of transcription (STAT family) are crucial components of diverse signal-transduction pathways that are actively involved in cellular survival, proliferation, differentiation, and apoptosis [1]. JAKs are constitutively associated with many cytokines and growth factor receptors [2]. Activation of JAKs is implicated in tumorigenesis, and genetic alterations of genes encoding JAK2 are partly responsible for the activation of the JAK/STAT signaling pathway. Currently, the occurrence of the *JAK2* V617F mutation is well recognized in chronic myeloproliferative disorders (MPDs). The G to T exchange at nucleotide 1849 in exon 14 of the *JAK2* gene leads to a substitution of valine with phenylalanine at the amino acid position 617 (V617F) of the JAK2 protein. Overexpression of *JAK2* V617F drives the constitutive kinase activity toward production of a protein segment containing the JAK2-activation loop [3]. The mutation is located in the JH2 pseudokinase domain of JAK2, which is involved in the autoinhibition of its kinase activity, and results in the constitutive activation of JAK2, which promotes erythropoietin hypersensitivity and growth-factor independence in transfected cell lines, in addition to inducing erythrocytosis in a murine transplant model. The frequency of the mutation is 65%–97% in polycythemia vera (PV), about 23%–57% in essential thrombocythemia (ET), and 35%–57% in myelofibrosis with myeloid metaplasia [3–7]. Detection of the JAK2 mutation has recently been included under the essential diagnostic criteria for PV by the World Health Organization (WHO) diagnostic criteria for PV [8].

High resolution melting (HRM) is rapidly becoming the most important mutation-scanning methodology that allows mutation scanning and genotyping without the need for costly labeled oligonucleotides. Evaluation of mutation scanning by HRM analysis has shown high sensitivity and specificity, concluding that HRM in the presence of a saturating dsDNA binding dye was a suitable and specific technique for mutation scanning. It is a closed-tube method,

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indicating that PCR amplification and subsequent analysis are sequentially performed in the well, making it more convenient than other scanning methodology. The aim of our study was to assess the value of the HRM analysis using real-time polymerase chain reaction (PCR) (Lightcycler® 480; Roche Applied Science) for identifying the *JAK2* V617F missense mutation. Additionally, HRM analysis was compared with amplification refractory mutation system (ARMS) assay.

2. Materials and methods

2.1. Patient samples and DNA extraction

The characteristics of 17 patients with MPD are shown in Table 1. Peripheral blood samples were obtained from 17 patients with MPD and 16 normal control subjects. This study included 7 PV patients (41.1%), 6 ET patients (35.3%), and 4 myelofibrosis patients (23.5%). Eleven patients with MPD harboring the *JAK2* V617F mutation, including 2 homozygous (18.2%) and 9 heterozygous (81.8%) mutants were found. The purified blood granulocytes were separated by density-gradient centrifugation using Ficoll-Paque PLUS (GE healthcare Bio-Sciences AB, Uppsala, Sweden). Subsequently, the DNAs of the blood granulocytes were extracted using the NucleoSpin® Blood Kit (Macherey-Nagel) according to the manufacturer's instructions. This study was approved by the Institute Review Board (IRB) of Kaohsiung Medical University Hospital.

2.2. Assay design and PCR conditions

Good amplicon design is essential to obtain robust and reproducible HRM analysis. Generally, it is recommended to use PCR products of smaller than 250 bp for best results. The difference between the melting curves of the wild-type and the heterozygote DNAs becomes smaller and difficult to differentiate if the product length increases. Moreover, extra care needs to be exercised in designing PCR reactions to avoid primer dimers and nonspecific amplification in HRM analysis. In this study, the primers for HRM analysis were selected using the Primer3 software and were as follows: forward (F): 5'-AGCAAGCTTTCTCACAAGCA-3' and reverse (R): 5'-CTGA-CACCTAGCTGTGATCCTG-3'. All the primers synthesized were all of standard molecular-biology quality (Protech Technology Enterprise Co., Ltd, Taiwan). The 155-bp PCR products with a single band were resolved on 2% agarose gels and visualized after staining with ethidium bromide (data not shown).

2.3. The HRM technique

PCR reactions were carried out in duplicate in 20 μl final volume using the LightCycler® 480 High-Resolution Melting Master (Reference 04909631001, Roche Diagnostics) 1× buffer—containing Taq polymerase, nucleotides and the dye ResoLight—and 30 ng DNA. The primers and $MgCl_2$ were used at a concentration of 2.5 mM, for detecting *JAK2* V617 missense mutation. The HRM assays were conducted using the LightCycler® 480 Instrument (Roche Diagnostics) provided with the software LightCycler® 480 Gene-Scanning Software Version 1.0 (Roche Diagnostics).

The PCR program required SYBR Green I filter (533 nm), and it consisted of an initial denaturation-activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C 15 s and elongation at 72 °C for 15 s with reading of the fluorescence; acquisition mode: single). The melting program included three steps: denaturalization at 95 °C for 1 min, renaturation at 40 °C for 1 min and subsequent melting that consists of a continuous fluorescent reading of fluorescence from 60 to 90 °C at the rate of 25 acquisitions per °C. The shapes of the difference–plot curves of the duplicate of each DNA sample must be reproducible both in shape and peak height.

2.4. Gene scanning

The melting-curve analysis carried out by the Gene-Scanning Software comprises of 3 steps: normalization of melting curves, equalizing the initial fluorescence and the fluorescence remnant after DNA dissociation to 100% and 0%, respectively; *shifting of the temperature axis* of the normalized melting curves to the point where the entire double-stranded DNA is completely denatured and finally, the difference plot analyzing the differences in the melting-curve shapes by subtracting the curves of the wild-type and *JAK2* V617 mutation DNAs: thus, the difference plot helps in the clustering of the samples into groups. To evaluate the discriminating power of each mutation in relation to the wild-type control DNA, the authors established the *resolution* which is defined as the difference between the relative fluorescence signals between the maxima of the peaks of the mutated and the wild-type DNAs in the difference plot.

Table 1
Characteristics of the 17 patients with myeloproliferative disorder.

Age (y), mean (range)	66.6 (36–80)
Male/female gender, *n* (%)	8/9 (47%/53%)
Red blood cell count ($\times 10^6$/μl), mean (range)	3.6 (2.18–5.65)
White blood cell count ($\times 10^3$/μl), mean (range)	9.6 (4.4–26.8)
Hemoglobin (g/l), mean (range)	12.3 (7.7–18.8)
Platelet count (10^3/μl), mean (range)	552.2 (156–1281)
Hematocrit (%), mean (range)	36.8 (24.0–51.5)

2.5. Direct DNA sequencing

To confirm the results of the HRM analysis, sequencing analysis was also carried out for all samples. After the HRM analysis, samples were purified using the PCR-M™ clean-up system (VIOGEN) and subsequently, the PCR products thus generated can be sequenced directly. The sequencing reaction was conducted in a 10-μL final volume using 5 μL of the purified PCR product, 2.5 μM of one of PCR primers and 1 μL of ABI-PRISM 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 310 Genetic Analyzer (Applied Biosystems).

2.6. Sensitivity of HRM analysis in identifying the JAK2 V617F missense mutation.

The authors mixed the wild-type DNA (G allele) with that of the mutant type (T allele) in different concentrations (0% mutant, 5% mutant, 10% mutant, 20% mutant, 30% mutant, 40% mutant, 50% mutant, 60% mutant, and 100% mutant) in order to evaluate the sensitivity of the methodology. The sensitivity test was carried out in duplicate for ensuring the reproducibility of the normalized and temperature-shifted difference plots.

2.7. Validation of the reproducibility of detecting JAK2 V617F missense mutation by HRM analysis

Identical DNAs (samples A and B) were analyzed repetitively by HRM for confirming whether the melting curve was reproducible using both normalized and temperature-shifted difference plots.

2.8. Validation of applicability of HRM for identifying JAK2 V617F missense mutation using different concentrations of DNA.

To validate the assay performance of the assay, the mutant DNA (heterozygous) was diluted to yield different concentrations including 100, 50, 40, 30, 20, 10, and 5 ng/μl.

2.9. JAK2 V617F genotyping by amplification refractory mutation system (ARMS)

PCR primers were earlier described [9]: forward outer (FO), 5'-TCCTCAGAACGTT-GATGGCAG-3'; reverse outer (RO), 5'-ATTGCTTTCCTTTTTCACAAGAT-3'; forward wild-type-specific (Fwt), 5'-GCATTTGGTTTTAAATTATGGAGTATaTG-3'; reverse-mutant-specific (Rmt), 5'-GTTTTACTTACTCTCGTCTCCACAaAA-3'. Amplifications were performed for 30 cycles with HotStar Taq polymerase (Qiagen, Crawley, UK), an annealing temperature of 60 °C, 25 ng genomic DNA, and standard amplification conditions, except that the final concentrations of the outer primers and the mutant/wild-type-specific inner primers were 1 μmol/l and 0.5 μmol/l, respectively. Products were resolved on 3% agarose gels and visualized after staining with ethidium bromide.

3. Results

3.1. Detection of JAK2 V617F missense mutation detection in patients with MPD using HRM analysis

In this study, we are able to identify the homozygous (T/T) and heterozygous (G/T) mutants could be differentiated from the wild-type DNA. In the normalized and temperature-shifted difference plots, the melting profile of a wild-type control (G/G) was chosen as the horizontal base line, and the relative differences in the melting of all the other samples were plotted relative to this baseline. Fig. 1 shows that the *JAK2* V617F missense mutation can be easily distinguished in the normalized and temperature-shifted difference plots.

Eleven out of 17 patients (64.7%) were positive for the presence of *JAK2* V617F mutation. The prevalence of mutation in the different subtypes of MPDs was 85.7% in PV (6 of 7 patients), 66.7% in ET (4 of 6) and 5.9% in myelofibrosis (1 of 4).