PCR-HRM for Detecting $JAK2V617F$ Gene Mutation: Is It a Sensitive Assay?

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Abstract

Background: A substitution of G to T at nucleotide 1849 in exon 14 of the Janus kinase2 ($JAK2$) gene is well recognized in myeloproliferative neoplastic disorders (MPNs). Based on WHO guidelines, detection of the mutation is very important to confirm the disease in suspected patients.

Methods: Eighty-seven patients with different background diseases were tested for $JAK2$ V617F mutation by four different methods, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), amplification refractory mutation system (ARMS), polymerase chain reaction-high resolution melting (PCR-HRM), and two different commercial kits.

Results: The mean age of patients was 53.38±17.43 years, 72.4% were males, and 37.6% were females. $JAK2$ mutation was detected in 16 patients (18.3%). Of those, 7 (43.75%) suffered from PV, 5 (31.25%) from ET, 3 (18.75%) from PMF, and 1 (6.15%) from unclassified neoplastic disorders. The frequency of $JAK2$ mutation was 71.4% (5/7) in PV, 80% (4/5) in ET, and 66.7% (2/3) in PMF patients. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and GE of PCR-HRM for detection of the $JAK2$ mutation was 86.7%, 100%, 100%, 97.3%, and 97.7%, respectively. While the sensitivity, specificity, PPV, NPV, and GE of PCR-RFLP were 93.3%, 80.5%, 50%, 98.3%, and 82.7%, respectively. On the other hand, the sensitivity, specificity, PPV, NPV, and GE of ARMS assays were evaluated by about 80%, 96%, 100%, 96%, and 96.5%, respectively.

Conclusion: This study showed that PCR-HRM was a more sensitive assay to detect the $JAK2$ V617F mutation than the other assays. So, it can be used as a quick, easy, and effective method for screening the $JAK2$ V617F mutation in patients with MPNs disorders. PCR-RFLP must accompany it as a gold standard method for confirmation of the mutation of $JAK2$ V617F.

Keywords: $JAK2V617F$ Mutation, PCR-HRM, PCR-RFLP, Myeloproliferative Neoplastic Disorders
INTRODUCTION:

The Janus kinase 2 (JAK2) is a critical component of diverse signal-transduction pathways. These molecules are involved in cellular survival, proliferation, differentiation, apoptosis, and cytokine signaling in normal and neoplastic cells through tyrosine kinase activity. The substitution of valine for phenylalanine at amino acid position 617 (V617F) of the JAK2 protein results in the activation of the JAK/STAT signaling pathway. Mutations in genes encoding JAK2 are, to some extent, responsible for tumorigenesis. Nowadays, the incidence of the JAK2 V617F mutation is well established in chronic myeloproliferative disorders (MPNs). The prevalence of the mutation is 65%–97% in polycythemia vera (PV), about 23%–57% in essential thrombocythemia (ET), and 35%–57% in primary myelofibrosis with myeloid metaplasia. One of the significant WHO criteria for the diagnosis of PV is the detection of JAK2 mutations. Therefore, reliable and sensitive methods are needed for the detection of these mutations.

Many molecular assays, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR), have been previously described for the detection of JAK2 V617F mutations. High resolution melting (HRM) is a rapid and cost-effective methodology for mutation scanning and genotyping. Its mechanism based on the chelating of dsDNA dye with double-strand DNA. PCR-HRM assay is a suitable and specific closed-tube technique for mutation scanning and makes it more convenient than other scanning methods. Our study aimed to evaluate the diagnostic value of the PCR-HRM analysis for identifying the JAK2 V617F missense mutation. Therefore, PCR-HRM analysis was compared with ARMS, PCR-RFLP, and commercial kits, as well as the clinical background of the patients. Then, we reported the frequency of JAK2 V617F missense mutation in Iranian patients with PV, ET, and PMF.

METHODS:

Patient samples and DNA extraction

Peripheral blood samples were obtained from 16 patients with MPD and 71 control subjects. Control subjects consisted of 27 patients (38.03%) with chronic diseases such as hypertension, 31 patients (43.66%) with lung disorders such as Asthma and COPD, 8 (11.27%) patients with infectious diseases such as tuberculosis and 5 normal subjects (7.04%) without any signs and symptoms of diseases. The demographic data of the subjects are summarized in Table 1. The study population consisted of 7 PV patients (43.75%), 5 ET patients (31.25%), 3 myelofibrosis patients (18.75%), and a patient with an undetermined myeloproliferative disorder (6.25%). DNAs of the blood leucocytes were extracted using the silica-based kit (Yekta Tajhiz, Iran) according to the manufacturer’s instructions. This study was approved by the National Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Iran.

The HRM technique and PCR conditions

A PCR reactions were carried out in 20 µl final volume using 1× buffer, containing Taq DNA polymerase, nucleotides, and the SYBR green dye, 2.5 mM of each primer (forward (F): 5’-AAGCTTTCTCACAAGCATTTGGTTT-3’ and reverse (R): 5’-AGAAAGGCATTAGAAAGCCTGTAGTT-3’) as well as MgCl2 and 30 ng DNA to amplify 155 bp of JAK2 gene harboring probably V617F mutation. The PCR program consisted of an initial denaturation-activation step at 95 °C for 15 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s and elongation at 72 °C for 10 s with the acquisition of the fluorescence dye at 533 nm at the end of the elongation step).
The melting program included a rising melting ramp by 0.1 degrees each level from 65 to 95°C that consists of a continuous fluorescent reading of fluorescence per °C. The PCR-HRM reactions were performed and analyzed with the Rotor-Gene real-time analyzer 6000™ (Corbett Life Sciences, Mortlake, Australia). Negative controls and positive controls were used as a reference curve to generate the difference plot (Fig 1).

**Direct DNA sequencing**

To confirm the results of the HRM analysis, sequencing analysis was carried out for 5 positive samples. The sequence detection was conducted using the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The graphs were analyzed by BioEdit v7.0.5.

**JAK2 V617F genotyping by ARMS**

ARMS-PCR was carried out by tetra primers earlier described: G49-JAK2-FO, 5'-TCCTCAGAACGTTGATGGCAG-3'; G50-JAK2-RO, 5'-ATTGCTTTCCTTTCACAAGAT-3'; G51-JAK2-FWT, 5'-GCAATTGTTTTTAAATTATGGGAGTATaTG-3'; G52-JAK2-RMT, 5'-GTTTTACTTACTCTCGTCTCACAaAAA-3'. Also, multiplex PCR was using three primers: G46-JAK2-AS-R, 5'-CTgAATAgTCCCTACAgTTTTCaTTCaTTCA-3'; G47-JAK2-AS-mtf, 5'-AGCaTTTggTTTTAAATTATgAgTATATT-3'; G48-JAK2-AS-CF, 5'-ATCTATAgTCTgCTgAAAgTAggAgAAAg-3' described by Baxter previously. Amplifications were performed for 30 cycles with 2X master mix (PARS Tous co, IRI) (consisting of 1X buffer, 0.2 uM of each dNTP, 1.5 µM MgCl2, and 1 U Taq DNA polymerase), 25 ng genomic DNA, and standard amplification conditions with an annealing tempera-
**Figure 1.** HRM curves of JAK2 exon 14 V617F missense mutation. A- A melting curve profile plot which synchronization samples in maximum and minimum fluorescence emission. B- Difference plot showed a characteristic melting curve and temperature shifted plot of mutant (T allele) and wild type (G allele). C- The result of HRM generated by Rotor-Gene 6000 Series Software 1.7- the mutant allele was confirmed by confidence more than 80%.
ture of 58 °C. However, 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 2% agarose gels and visualized after staining with safe stain (Fig 2).

**JAK2 V617F genotyping by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)**

To detect the JAK2V617F mutation by restriction digestion, a portion of the JAK2 region that acquires this mutation (462 base pairs) was amplified by using two forward and reverse outer primers (G49-JAK2-FO and G50-JAK2-RO), as described above. The amplified fragment was digested with the BsaXI restriction enzyme (BioLabs™, New England) as the manufacturer’s instruction. The final digested products were visualized on 2% stained agarose gels with safe stain. The undigested fragment implicated JAK2 V617F missense mutation because of losing the restriction enzyme site but the wild type cases were digested and produced two visible bands (190, 242 and 30bp which is disappear on agarose gel). Logically, existing the undigested bands plus digested one indicated the heterozygosity may be due to the low allele burden of mutation in the background of wild type allele or due to incomplete endonuclease activity (Fig 3).

**Statistical analysis**

The Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL), version 22.0, and software was used for statistical analysis. Both HRM results and PCR findings were cross compared with the results of the myeloproliferative disease and PCR-RFLP (as the gold standard)
and accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV) of each test are reported.

**Ethical Approval**

The work has been approved by the ethical committees of National Research Institute of Tuberculosis and Lung Diseases (NRITLD). Written informed consent was obtained from all participants to enter the study.

**RESULTS:**

In this study, HRM assay could be able to differentiate the homozygous (T/T) mutant from the wild-type DNA in comparison with the melting profile of wild-type control (G/G). Thirteen out of 16 MPNs patients (81.25%) were positive for the presence of JAK2 V617F mutation by HRM assay. The prevalence of the mutation in the different subtypes of MPNs was 71.4% in PV (5 of 7 patients), 80% in ET (4 of 5) and 100% in myelofibrosis (3 of 3). The background disease of one patient was not determined. She was suspected of MPN disorders but the WHO criteria were not confirmed in this patient (Table 2). This study showed that the sensitivity of HRM analysis to detect the JAK2 V617F mutation in patients with MPNs and those with suspected MPNs was 86.6% vs 93.3% for PCR-RFLP and 80% for PCR-ARMS assays. The sensitivity of commercial kits (ipsogen® JAK2 MutaQuant® Kit CE, Qiagen from Germany and JAK2 RG version 2, Novin gene from Iran) was lower than homemade assays (66.7%) (Table 3).

**DISCUSSION**

The objective of this study was to evaluate the efficacy of several JAK2 V617F mutation PCR-based assays. The result should be used in selecting an appropriate method so that both patients and the laboratories are involved in its benefit. Numerous techniques include, allele-specific polymerase chain reaction (AS-PCR), genomic DNA-PCR-sequencing, PCR-amplification refractory mutation system, PCR-restriction analysis and DNA-melting curve analysis, have been developed for the detection of the JAK2 V617F missense mutation during the last decades. Each of them has its advantages and disadvantages: for example, in spite of the power of DNA sequencing to detect any specific mutation, its sensitivity is low and the cost is high. On the other hand, PCR-ARMS, which is introduced as one of the most sensitive (0.01%–5%) techniques needs post-PCR manipulation. The false-positive results of PCR-RFLP are due to incomplete digestion with the restriction enzyme can be complicated the interruptions and on the other hand, this technique is time-consuming and expensive. Many important parameters such as sensitivity, specificity, the cost of a test should always be considered to evaluate and select these methods. There are very few studies for evaluat-
Table 2. Comparison of \( \text{JAK2}V617F \) mutation positive samples as assessed by the PCR-based assays.

<table>
<thead>
<tr>
<th>Disorders</th>
<th>No</th>
<th>PCR-RFLP‡</th>
<th>PCR-ARMS¶</th>
<th>PCR-HRM§</th>
<th>Commercial kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycythemia Vera</td>
<td>7</td>
<td>7 (100%)</td>
<td>5 (71.4%)</td>
<td>5 (71.4%)</td>
<td>4 (57.1%)</td>
</tr>
<tr>
<td>Primary Mielofibroses</td>
<td>3</td>
<td>2 (66.7%)</td>
<td>2 (66.7%)</td>
<td>3 (100%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Essential Thrombocytopenia</td>
<td>5</td>
<td>4 (80%)</td>
<td>4 (80%)</td>
<td>4 (80%)</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Clinically suspected MPNs*</td>
<td>1</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>14 (87.5%)</td>
<td>12 (75%)</td>
<td>13 (81.25%)</td>
<td>10 (62.5%)</td>
</tr>
</tbody>
</table>

* Myeloproliferative neoplasms.
‡ Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
¶ both multiplex Allele specific and amplification-refractory mutation system
§ Polymerase Chain Reaction-High Resolution Melt analysis
† Two samples were heterozygote (\( \text{JAK2}V617F \) mutation was not confirmed by DNA sequencing)

Table 3. Sensitivity and Specificity of different assays to detect \( \text{JAK2}V617F \) mutation in the confirmed and suspected MPNs patients

<table>
<thead>
<tr>
<th>Disorders</th>
<th>PCR-HRM§</th>
<th>PCR- RFLP‡</th>
<th>ARMS-PCR¶</th>
<th>Commercial Real-time kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>86.6%</td>
<td>93.3%</td>
<td>80%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>80.5%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>100%</td>
<td>50%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>97.3%</td>
<td>98.3%</td>
<td>96%</td>
<td>90.9%</td>
</tr>
<tr>
<td>General efficacy</td>
<td>97.7%</td>
<td>82.7%</td>
<td>96.5%</td>
<td>92.3%</td>
</tr>
</tbody>
</table>

§ Polymerase Chain Reaction-High Resolution Melt analysis
‡ Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
¶ both multiplex Allele specific and amplification-refractory mutation system
PCR-HRM and JAK2 V617F mutation

...ing the currently available testing assays for detecting the JAK2 V617F mutation in Iranian MPNs patients. Some studies showed that there is a good concordance among PCR-HRM with three other screening methods (ARMS-PCR, PCR-RFLP, and quantitative method) for JAK2 V617F detection in MPNs patients especially when there is a higher concentration of the mutant alleles. In our study, four different screening methods were assessed using a total of 16 confirmed MPNs out of 87 patients with different symptoms of diseases such as chronic lung disease, infectious and/or solid cancer disorders. The assays were compared with the PCR-RFLP method, a widely used technique, as a reference assay for the detection of the JAK2 V617F mutation allele. The statistical analysis showed a good concordance among all assays (P=0.000) but the melting curve assay proved less reliable at lower levels of JAK2 V617F mutation allele (Table 2). A serial dilution of positive samples showed that the PCR-HRM assay could detect the mutant allele by lowers than 0.1×10^-4 ng/μl of pool genomic DNA. Therefore the burden of the mutant allele in PMF patients who were positive with PCR-HRM probably was lower than the limit of detection of mutation in wild type background by PCR-RFLP assay (Table 2). A second, smaller fragment in addition to the complete band in mutant allele while in allele specific method two different specific primers could detect the wild type and mutant alleles. The role of the two outer primers was to detect the PCR inhibitor as an internal control. In cases with the presence of JAK2 mutation, annealing with intermediate primer will happen in multiplex ARMS-PCR assay and consequently, the sideband will be observed. In Allele-specific methods, the mutation was detected if the mutant allele specific primers were annealed and produced a 240bp fragment (Fig 2). Both of the ARMS PCR methods need post-amplification manipulation which can cause cross-contamination. It is a major disadvantage of these methods. For this reason and because of the PCR-HRM method’s advantages over PCR-ARMS such as its ease of performance and speed of it as well as its high sensitivity compared to other molecular assays, we think PCR-HRM may be a good tool for screening of JAK2 V617F mutation in clinical suspension patients.

Our evaluation revealed that the two commercial kits for the detection of JAK2 V617F missense mutation were less sensitive methods. The limit for mutation detection by them was subjective and may depend on the burden level of the allele mutation. Unfortunately, the Iranian manufacturer did not specify the limit allele burden. Maybe the allele burden of the commercial kit was more than 20% mutant DNA in the background of the wild type allele which is higher than the HRM-PCR assay. The limit of detection (LOD) of the ipsogen® JAK2 MutaQuant® Kit was 0.042% which is lower than ARMS assays. On the other hand, based on ipsogen® JAK2 MutaQuant® Kit Handbook, in case of additional mutations located in nucleotides 88504 to 88622, the result of test can report false negative. Another study showed the low sensitivity of the ipsogen® JAK2 MutaQuant® Kit in detection of JAK2 V617F mutation. Since the accessibility of the ipsogen® JAK2 MutaQuant® Kit is not easy in our country as well as its lower sensitivity to detect JAK2 V617F mutation we...
recommend the PCR-HRM assay as a substitution assay to detect the mutation.

CONCLUSION
In conclusion, we have compared the results of four PCR-based assays including PCR-RFLP for detection of the JAK2 V617F mutation in blood samples from clinically confirmed and suspected patients with MPNs disorders. Our results have demonstrated that the PCR-HRM protocol is a quick, easy, and effective method to screen the JAK2V617F mutation in patients with MPNs disorders. And for confirmation of the result, it must be accompanied by PCR-RFLP as a gold standard method for the detection of the mutation of JAK2 V617F.

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REFERENCES