Comparisons of ARMS-PCR and AS-PCR for the Evaluation of JAK2V617F Mutation in Patients with non-CML Myeloproliferative Neoplasms

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Abstract
Background and objectives: JAK2 is a nonreceptor tyrosine kinase that plays a major role in myeloid disorders. JAK2V617F mutation is characterized by a G to T transverse at nucleotide 1849 in exon 12 of the JAK2 gene, located on the chromosome 9p, leading to a substitution of valine to phenylalanine at amino acid position 617 in the JAK2 protein. In this study we compared two molecular methods namely ARMS-PCR and AS-PCR for the evaluation of JAK2V617F mutation in patients with myeloproliferative neoplasms.

Material and methods: In this study we evaluated JAK2 mutation in 89 patients with Myeloproliferative neoplasm (MPNs) by simple randomized sampling. The mutation was detected by ARMS-PCR and AS-PCR in patients. Three DNA samples were sequenced for confirmation of the above techniques.

Results: The JAK2 V617F mutation was detected in 86.6% (26/30) of patients with polycythemia vera and 61.5% (8/13) of patients with idiopathic myelofibrosis. None of 31 CML patients were detected by ARMS-PCR and AS-PCR. In essential thrombocytopenia using ARMS-PCR and AS-PCR 46.6% (7/15) and 53% (8/15) of patients were positive, respectively. The mutation was confirmed by sequencing.

Conclusions: The results of the study showed that similarity with other studies by two techniques and detection of the JAK2V617F mutation may depend on the molecular technique used. Also, JAK2 mutation detection is an appropriate tool for differential diagnosis of non-CML myeloproliferative neoplasms from benign condition like reactive erythrocytosis and thrombocytosis.

Key words: JAK2V617F mutation, myeloproliferative neoplasms, ARMS-PCR, Allele-specific PCR

Introduction
JAK2 V617F mutation in chronic myeloproliferative neoplasm was discovered by different methods.1,4) JAK2 is a cytoplasmic tyrosin kinase with a key role in a different signal transduction pathways like interleukin 3, 5 (IL3, IL5) and granulocyte-monocyte-colony stimulation factor (GM-esf).5, 6) JAK2 contains four major function domains, one tyrosin kinase domain (JH1), one psudo kinase domain(JH2), one sic-homology2 (SH2) domain and the FERM domain.
Identification of the JAK2 mutation is new discovery in the field of chronic myeloproliferative neoplasm-(MPNS).7, 8) Different techniques have been used for the detection of this mutation, such as genomic DNA-PCR-sequencing, RT-PCR, PCR-ARMS (amplification refractory mutation system), Allele-specific PCR, PCR restriction analysis, and real-time PCR.9) In this study frequency of JAK2 mutation, in patients with essential thrombocytopenia (ET), polycythemia vera (PV) and idiopathic myelofibrosis (IMF) and chronic myelogenous leukemia (CML) was studid using two methods (i.e. AS-PCR and ARMS-PCR) in order to find the most sensitive method for the detection of this mutation.
Materials and Methods
We evaluated 89 previously treated and newly diagnosed patients with MPDs and 50 normal control samples: 30 patients with PV, 13 patients with IMF, 15 patients with ET and 31 patients with CML. Fifty patients were male (56.6%) and 39 were female (43.4%). The mean age was 48 ranging from 16 to 76 years.
The patients were selected from outpatient clinic of Bone Marrow Transplantation Center of Shariati Hospital and the Oncology Center of Imam Khomeini Hospital. The mutation was detected by two methods, AS-PCR and ARMS-PCR on mononuclear cell.(9)

1-AS-PCR method: SsRNA was extracted from 5ml of fresh peripheral blood in EDTA with standard trizol method.(11)
Extracted ssRNA was measured by OD, absorbance in 260nm and ssRNA concentration was calculated using the formula below:

\[
\text{[SSRNA]}(\text{mg/ml}) = \frac{A_{260} \times 40 \text{ mg/ml} \times \text{dilution factor}}{\text{OD}}
\]

AS-PCR is a common PCR technique widely used to detect known mutation based on the amplification of mutant alleles using mutant specific primer. This method was first reported by baxter et al.(10) and sequences of the primers used in this study are given below:(10)
Forward 5’-GAAGATTGTATTTAATGAAAGCCTT-3’
Reverse 5’-GTAATACTAATGCGCAGTACACTAAGTT-3’
Mutant 5’- AGCATTGTTTTAAATTAGGATATATT-3’

PCR parameters were as follows: Denaturation for 5 minutes at 95°C, for one cycle; 36 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 40 seconds, elongation at 72°C for 45 seconds, hold for 10 minutes at 72°C, and infinite hold at 4°C.
A 488-bp product was obtained from both wild type and mutant alleles, while a 295 bp product indicated the presence of the mutation allele.(4) Of course the AS-PCR was not designed to distinguish zygosity. The zygosity was determined by RFLP-PAGE (restriction fragment length polymorphism/ ployacrilamide gel electrophoresis) sample interpretation.(11)

2-ARMS method: Genomic DNA was extracted from 5ml of fresh peripheral blood in EDTA using proteinase K method. Primers used in ARMS-PCR method include:

Forward Outer (FO): 5’-TCCTCAGAACGTTGATGGCAG-3’
Reverse Outer (RO): 5’-ATTGCCCTCCTTTTCACAAGAT-3’
Forward wild-type specific (FWt): 5’- GCATTGTTTTAAATTAGGATATATG-3’
Reverse mutant-specific (RMt): 5’- GTTTTACTCTCCTCCTCCACAAA-3’
We used 25ng of genomic DNA, 0.5μl of each FO, RO and FWt primers and 1μl of RMt primer. DNA was amplified in a 40-cycle PCR reaction. The PCR products were analysed on 3% TBE agarose gels. The FO and RO primers would amplify a 463-bp product from JAK2 gene, while the primer RO and FWt primers would amplify a 229-bp product as wide type alleles and also amplify a 279-bp product from FO and RMt primers.

Results
In this study we compared the results of two methods, ARMS-PCR and AS-PCR.
Result of ARMS-PCR showed that JAK2 mutation was positive in 26 out of 30 PV patients (86%), 8 out of 13 IMF patients (61%), 7 out of 15 ET patients (46.6%) and none of the 31 CML patients (Figure- 1, Table-1). Using AS-PCR method, we observed that 26/30 PV patients (86%), 8/13 IMF patients (61%), 8/15 ET patients (53%) and also none of 31 CML patients were positive for JAK2 V617F mutation (Figure- 2, Table-1).
All mutations were confirmed by sequencing purified PCR product by Millegen Company (Germany).

<table>
<thead>
<tr>
<th>Disease</th>
<th>JAK2V617F positive</th>
<th>JAK2V617F positive</th>
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<tbody>
<tr>
<td>PV</td>
<td>86%</td>
<td>86%</td>
</tr>
<tr>
<td>ET</td>
<td>53.3%</td>
<td>46%</td>
</tr>
<tr>
<td>IMF</td>
<td>61.5%</td>
<td>61.5%</td>
</tr>
<tr>
<td>CML</td>
<td>14%</td>
<td>14%</td>
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Figure- 1. ARMS-PCR assay for detection of JAK2V617F mutation; lane1, 2 positive control, lane3, 4, 5, 6, 8 mutation negative patients, lane7 mutation positive patients, lane 9 negative control, lane 10, 50bp DNA ladder.
Discussion
JAK2 V617F mutation, in chronic myeloproliferative neoplasm, was investigated by different methods.(1&4) Identification of the JAK2 mutation is new discovery in the field of chronic myeloid proliferative neoplasm (MPNS).(7&8) Different techniques have been used for this detection of the mutation, such as genomic DNA-PCR-sequencing, RT-PCR, and ARMS-PCR, AS-PCR, PCR restriction analysis, and real-time PCR.(9)
In one study, the prevalence of mutation in PV patients has been found to be 81% (58/72), in ET patients 41% (24/59) and in IMF patients 43% (15/53).(11) In Baxter et al's study in 2005 using mutation specific PCR method,(4) the JAK2 mutation detected in 71 of 73 (97%) patients with PV, 29 out of 51 (57%) with ET, and 8 out of 16 (50%) with IMF.
In support of previous reports, we observed the mutation in 86% of PV patients which is comparable with result of James' (i.e. 86%), (1) Jelinek's (86%) (14) and Jones's studies (81%).(11) The highest rate of mutation (97%) has been reported by Lippert's group using allele-specific quantitative polymerase chain reaction (qPCR) (15) and the lowest one (65%), has been reported by Kralovics's group using DNA sequencing and microsatellite mapping.(2) The highest detection rate in IMF was found by Jelinek (14) with a frequency 95% (18 of 19 patients) who used pyrosequencing method and the lowest observation of mutation with 35% frequency (16 of 46 patients) detected in Levin study.(14) In a study by Campbell et al (25) on 806 ET patients by AS-PCR techniques, 414 cases (53.4%) were positive and 362 (46.6) were negative for this mutation.
Comparisons of the sensitivity of the different techniques for JAK2V617F mutation, show that AS-PCR techniques compared to other methods like RFLP, pyrosequencing and ARMS are more sensitive.(13) The JAK2 mutation has not been found in our 31 CML patients, which is the same as Jones' group study,(13) however, in other study JAK2 mutation and abl-bcr translocation were detected in some patients who had received Imatinib treatment.(16) Moreover, the JAK2 mutation was detected by ARMS-PCR. This result shows that AS-PCR is more sensitive than ARMS-PCR. Probably, the main cause of this difference that we observed between the two molecular techniques is the sensitivity of this method.
In summary, we have shown that a single acquired point mutation in JAK2 is present in virtually most patients with PV and in about half of those with either ET or IMF. Thus, the detection of mutation could be used not only as a diagnostic tool, but also for the classification and management of patients with MPDs. Also, there were no significant differences between the two methods and objectively the AS-PCR is a little more sensitive than ARMS-PCR.

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References