

JAK2 p.V617F mutation – tetra-primer PCR and PCR-RFLP comparative semiquantitative approaches for estimation of the mutant allele in myeloproliferative neoplasms

Mutația JAK2 p.V617F – tehnicile tetra-primer PCR și PCR-RFLP ca metode comparative semicantitative de apreciere a alelei mutante în neoplasmele mieloproliferative

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Abstract

Polycythemia vera, essential thrombocythemia and primary myelofibrosis are the three typical BCR-ABL negative myeloproliferative neoplasms. Recent studies indicated that a somatic single-point mutation, JAK2 p.V617F is a crucial molecular event in the pathogenesis of these diseases. Taking into consideration the importance of this mutation as a diagnosis and prognosis marker, we adapted in our laboratory a tetra-primer polymerase chain reaction (tetra-primer PCR) and a polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) previously described assays for detection of JAK2 p.V617F. Meanwhile, we built a dilution based scale of mutant p.V617F homozygous DNA in wild-type homozygous DNA which serves for a semiquantitative estimation of the mutant allele proportion. Both techniques were shown to be similar sensitive: the lower detection limit of mutant allele was around 2% (tetra-primer PCR) and around 3% (PCR-RFLP); however the tetra-primer PCR has the advantage over the PCR-RFLP of being a more rapid assay. We successfully managed to implement tetra-primer PCR and PCR-RFLP assays for JAK2 p.V617F analysis and to provide meanwhile semiquantitative approaches for estimation of the mutant allele in myeloproliferative neoplasms.

Keywords: myeloproliferative neoplasms, JAK2 p.V617F, tetra-primer PCR, PCR-RFLP, semiquantitative approach

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Rezumat

Policitemia vera, trombocitemia esențială și mielofibroza primară sunt cele mai reprezentative neoplasme mieloproliferative negative pentru fuziunea BCR-ABL. Studii recente au demonstrat că o mutație punctiformă somatică, respectiv JAK2 p.V617F, reprezintă un eveniment molecular esențial în patogeneza acestor boli. Având în vedere importanța acestei mutații ca un marker diagnostic și de prognostic în cele trei neoplasme mieloproliferative, am adaptat în laboratorul nostru două tehnici descrise anterior pentru detecția mutației JAK2 p.V617F, respectiv tetra-primer polymerase chain reaction (tetra-primer PCR) și polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP). În același timp, pentru estimarea semicantitativă a proporției alelei mutante, am construit o scală bazată pe diluții succesive ale unei probe ADN provenind de la un individ homozigot pentru alela p.V617F într-o probă ADN provenind de la un individ homozigot pentru alela normală. Am demonstrat că ambele tehnici au o sensibilitate asemănătoare: limita de detecție a alelei mutante a fost de aproximativ 2% (tehnica tetra-primer PCR) și respectiv 3% (tehnica PCR-RFLP). Totuși, tehnica tetra-primer PCR are avantajul de a fi o tehnică mai rapidă decât PCR-RFLP. Astfel, am reușit să implementăm cu succes tehnicile tetra-primer PCR și PCR-RFLP în laboratorul nostru, vizând detecția mutației JAK2 p.V617F și de asemenea am adaptat aceste tehnici pentru estimarea semicantitativă a alelei mutante în neoplasmele mieloproliferative.

Cuvinte-cheie: neoplasme mieloproliferative, JAK2 p.V617F, tetra-primer PCR, PCR-RFLP, estimare semicantitativă

Introduction

Chronic myeloproliferative diseases (CMPDs) are clonal hematopoietic stem cell disorders characterized by proliferation of one or more myeloid cell lineages in the bone marrow and increased numbers of mature and immature cells in the peripheral blood (1). The 2008 World Health Organization (WHO) classification of these disorders replaced the term "CMPDs" with "myeloproliferative neoplasms" (MPNs), in order to highlight their malignant origin. The 2008 classification of MPNs has incorporated new criteria based on the molecular pathogenesis markers recently discovered in these disorders (2).

Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are the three *BCR-ABL* negative classic MPNs. Although clinicopathologically distinct, the discovery of a single molecular marker common to PV, ET and PMF proved that these three MPNs are indeed related. This molecular marker is a single-point somatic mutation, specific to the myeloid hematopoietic precursors, a substitution guanine to thymine at

position 2343 (g.2343G>T) in the gene encoding the Janus kinase 2 (JAK2), which predicts a substitution valine to phenylalanine at position 617 (p.V617F) of the JAK2 molecule, in its pseudokinase domain (JH2) (3). Several studies published in 2005 indicated that this mutation leads to constitutive activation of JAK2 and thus to continuous activation of JAK-STAT signaling pathway (3-6). These studies and others which followed demonstrated the widespread occurrence of JAK2 p.V617F in MPNs: most of PV and about half of ET and PMF cases are JAK2 p.V617F positive.

Given its importance in the management of MPNs cases, testing for JAK2 p.V617F mutation became rapidly available in different genetic laboratories. Several techniques have been described, but here in this report we present the optimization in our laboratory of two sensitive and reproducible techniques, tetra-primer PCR and PCR-RFLP. As the mutation load seems to have importance in the prognosis and monitoring of the MPNs, we also developed semiquantitative approaches for estimation of the mutant allele proportion in peripheral whole blood samples.

Material and methods

Dilution of JAK2 p.V617F homozygous DNA in wild-type homozygous DNA

DNA obtained from peripheral whole blood with a commercially available kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) from a PV patient homozygous for *JAK2* mutant allele with minimal wild-type allele activity and from a healthy individual homozygous for *JAK2* wild-type allele were spectrophotometrically measured (Bio-Photometer plus, Eppendorf, Germany) and then brought to the same DNA concentration of 75 ng/μl. *JAK2* homozygous for the mutant allele DNA was then serially diluted in *JAK2* homozygous for the wild-type allele DNA, using a dilution factor of 1:2. In this way, 6 aliquots were obtained in which the proportion of mutant allele was 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 respectively, all of them with a constant DNA concentration of 75 ng/μl.

Tetra-primer PCR technique

The protocol described originally by Jones et al (1) was adapted to the conditions of our laboratory. This technique is based on the amplification of a control fragment from the *JAK2* gene, using two outer primers which are gene-specific, giving rise to an amplicon of 463 bp. Additionally, two primers which are allele-specific, a forward wild-type specific and a reverse mutant specific, combines with the corresponding outer primers to amplify the allele-specific fragment. The mutant specific and the wild-type specific fragments have 279 bp and 229 bp, respectively. In this way, both the wild-type and the mutant alleles can be studied in the same reaction. The PCR reactions were carried out in an Eppendorf thermocycler (Master-Cycler Gradient, Eppendorf, Germany), in 25 μl reaction volumes, with the following composition: 12.5 μl 2xPCR Master Mix containing recombinant Taq-DNA polymerase 0,05U/μl, MgCl₂ 4 mM, dNTPs mix 0,4 mM each (Fer-

mentas MBI, Vilnius, Lithuania), 10 pmoles of forward and reverse outer primers, 8 pmoles of wild-type and mutant alleles specific primers (Eurogentec, Belgium), 75 ng DNA and free nucleases water to 25 μl. Cycling conditions were as follows: initial denaturation at 94°C for 7 min, followed by 33 cycles of denaturation at 94°C for 35s, annealing at 57°C for 40s, extension at 72°C for 45s and a final extension at 72°C for 7 min. The PCR products were run in a 3% MetaPhor agarose (Lonza, Rockland, ME, USA) gel electrophoresis, stained with ethidium bromide and then visualized under an UV light transilluminator.

PCR-RFLP technique

This approach is based on a nested-PCR, which amplifies the *JAK2* region containing the p.V617F mutation, and subsequent digestion with *Bsa*XI restriction enzyme, after a protocol described by Horn et al (7). The first round of PCR amplification was performed in 25 μl reaction volumes, using 10 pmoles of each outer forward and reverse primers; the concentrations of the other PCR reagents were the same as described above for tetra-primer PCR technique. The second round of PCR amplification employed 2 μl of the first round PCR amplification, which served as amplification template, 10 pmoles of each inner forward and reverse primers and the other PCR reagents as described above. For the first round of amplification, the PCR program was: initial denaturation at 95°C for 7 min, followed by 35 cycles each consisting in denaturation at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 30s and a final extension at 72°C for 5 min. The PCR program for the second round of amplification was the same, except the annealing temperature, which was at 60°C instead of 55°C and the cycles number, which was 25 instead of 35. 8 μl of the second round PCR amplicons were digested overnight at 37°C with 2U of the restriction enzyme *Bsa*XI (New England Biolabs, Beverly, MA, USA). The digested PCR products were run in a 3% MetaPhor agarose

gel electrophoresis, stained with ethidium bromide and then visualized under an UV light transilluminator. If only normal allele is present in the sample, 2 bands are observed after digestion with *Bsa*XI: 170 bp and 203 bp, respectively. *JAK2* p.V617F abolishes this restriction site, the digestion products corresponding to the mutant allele appearing as a single band of 373 bp. The primers sequences used for both tetra-primer PCR and PCR-RFLP techniques were described elsewhere (1,7).

Results

Figures 1 and 2 present the electrophoresis obtained for the analysis of *JAK2* p.V617F mutation employing tetra-primer PCR and PCR-RFLP techniques, respectively, based on serial dilutions of mutant homozygous DNA in wild-type homozygous DNA. In the case of tetra-primer PCR, traces of the mutant allele were observed even at a dilution of 1/64 of the mutant homozygous DNA in wild-type homozygous DNA, while in the case of PCR-RFLP, a restriction pattern concordant with the mutant allele was observed after a dilution of 1/32 of the mutant homozygous DNA in wild-type homozygous DNA.

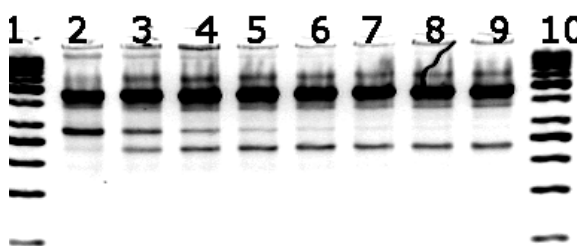


Figure 1. Dilution scale for *JAK2* p.V617F tetra-primer PCR method. 3% agarose gel. Lanes 1 and 10: 50bp molecular weight DNA marker; lane 2: *JAK2* p.V617F homozygote with minimal residual wild-type allele; lane 3: 1/2 dilution; lane 4: 1/4 dilution; lane 5: 1/8 dilution; lane 6: 1/16 dilution; lane 7: 1/32 dilution; lane 8: 1/64 dilution; lane 9: wild-type allele homozygote

Discussion

According to the 2008 WHO classification and management of the MPNs, testing for *JAK2* p.V617F mutation from peripheral blood white cells should be among the initial steps in the suspected cases of PV, ET and IMF. Moreover, demonstrating this mutation is considered to be a major diagnostic criterion, since its presence excludes a reactive myeloproliferation (2).

Most of the MPNs *JAK2* positive samples contain a mixture of mutant and normal clones in variable proportion. Homozygous mutations are more frequently encountered in PV and PMF than in ET and seem to be associated with a longer and more aggressive course of the disease; also, the mutation load seems to correlate with some complications of MPNs, such as thrombosis, myelofibrosis or leukemic transformation (4,8). Moreover, recent researches indicate that the *JAK2* p.V617F positive clone tends to decrease under cytoreductive treatment (9). Taking into consideration these aspects, a quantification of the mutant allele could be a reasonable approach.

Several techniques, such as pyrosequencing, real-time PCR or dHPLC have been successfully employed for an exact detec-

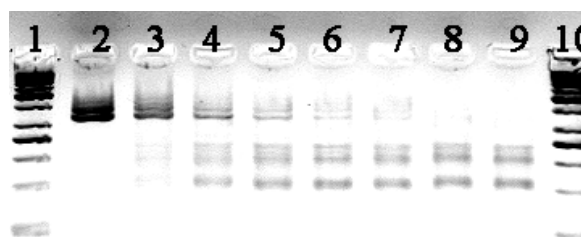


Figure 2. Dilution scale for *JAK2* p.V617F PCR-RFLP method. 3% agarose gel. Lanes 1 and 10: 50bp molecular weight DNA marker; lane 2: *JAK2* p.V617F homozygote with minimal residual wild-type allele; lane 3: 1/2 dilution; lane 4: 1/4 dilution; lane 5: 1/8 dilution; lane 6: 1/16 dilution; lane 7: 1/32 dilution; lane 8: 1/64 dilution; lane 9: wild-type allele homozygote

tion and quantification of the *JAK2* mutant allele (10). Although these techniques are accurate and elegant, they require expensive equipments and reagents, which might not be routinely affordable in all laboratories. By contrast, tetra-primer PCR and PCR-RFLP techniques are less expensive, requiring standard PCR instruments and provide sufficient sensitivity for routinely diagnosis purposes.

As a comparison between the sensitivity of the two methods, we observed similar lower detection limits of *JAK2* mutant allele of around 2-3% for both of them. Given their similar sensitivity, both the techniques, tetra-primer PCR and PCR-RFLP, can be used alternatively for studying *JAK2* p.V617F mutation, especially if appropriate positive and negative controls are used together with the samples to analyze. However, PCR-RFLP suppose two consecutive round of amplification plus a digestion step before the electrophoresis, while tetra-primer PCR suppose an amplification followed directly by electrophoresis. Thus, the tetra-primer PCR has the advantage over PCR-RFLP of being a more rapid assay, which employs a single reaction tube. Especially if a rapid diagnosis is required, tetra-primer PCR technique would be more desirable, because it is less time-consuming than PCR-RFLP technique.

Conclusions

In conclusion, we optimized in our laboratory two previously described protocols, a tetra-primer PCR and a PCR-RFLP respectively, for studying the *JAK2* p.V617F mutation, by which, to our knowledge, we successfully implemented for the first time in Romania the molecular analysis for this mutation. In our laboratory, these methods were shown to have the lower detection limit of mutant allele of around 2% (tetra-primer PCR) and around 3% (PCR-RFLP). These techniques are useful not only for diagnosing the *JAK2* p.V617F mutation, but also for estimating the mutation load

and monitoring the evolution of the mutant clone in time, based on the serial dilution scales we built up. Now that molecular analysis for *JAK2* p.V617F mutation is available in our country as well, the patients suspected of MPNs can be correctly investigated, taking into consideration that demonstrating *JAK2* p.V617F mutation became a major diagnostic criterion. As new molecular markers are discovered in hematological disorders, efforts must be made to their evaluation become part of the investigation protocol, in order to achieve a correct diagnosis and an appropriate further therapeutic management.

List of abbreviations

JAK2= Janus kinase 2
 PV= polycythemia vera
 ET= essential thrombocythemia
 PMF= primary myelofibrosis
 CMPDs= chronic myeloproliferative diseases
 WHO= World Health Organisation
 MPNs= myeloproliferative neoplasms
 tetra-primer PCR= tetra-primer polymerase chain reaction
 PCR-RFLP= polymerase chain reaction - restriction fragments length polymorphism
 DNA= deoxyribonucleic acid

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