Fast and reliable mutation detection of the complete exon 11–15 JAK2 coding region using non-isotopic RNase cleavage assay (NIRCA)

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Abstract

The screening for JAK2 V617F mutation in patients with polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis offers crucial information for the final diagnosis of these disorders. Recently, several JAK2 exons 12 and 14 mutations have been detected in V617F-negative patients with idiopathic erythrocytosis. The need for a rapid and accurate assay for the mutation screening in both exons 12 and 14 prompted us for the application of a method for the analysis of the entire coding region between exons 11 and 15. We applied the non-isotopic RNase cleavage assay and the accuracy of the method was verified in a series of V617F-positive, V617F-negative patients and healthy individuals, with no false results. This method can be applied in any laboratory without the requirement of specific sophisticated equipment.

Key words JAK2; myeloproliferative disorders; NIRCA

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Polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF) belong in a group of chronic hematologic diseases, called myeloproliferative disorders (MPD), which arise from the clonal expansion of a multipotent hematopoietic stem cell (1–3). Except from the common phenotypical characteristics, these disorders share genotypic similarities as 95% of PV patients and approximately 50% of ET and IMF patients exhibit gain-of-function V617F mutation of the Janus kinase (JAK) 2 gene (JAK2) (4–6). The 2008 WHO revised criteria for the diagnosis of PV, ET, and IMF employ JAK2-V617F mutation or similar mutation as a major criterion for the diagnosis of these disorders (7). As a result, the identification of this mutation provides a useful diagnostic tool and tends to become a standard diagnostic process in the evaluation of MPD.

Recently, novel mutations in the exon 14 (8–10), 13, 15 (10), and several exon 12 mutations (10–13) of JAK2 have been identified in JAK2-V617F negative patients with PV and idiopathic erythrocytosis. In addition, there is low JAK2-V617F neutrophil allele burden in ET patients, which may lead to a false negative analysis (14). As a result, an inexpensive, accurate and rapid assay that could be used in any laboratory and detect all possible mutations in exons 12–15 would be helpful in the diagnosis of these disorders and other clinical entities.

In this study, we report the application of a convenient and accurate method for the analysis of the coding region of the JAK2, which includes all the potential mutations identified in exons 12–15 up to now (exon 11–15 coding region), using the non-isotopic RNase cleavage assay (NIRCA). The technique was applied in a cohort of previously genetically characterized MPD patients and healthy individuals.

Materials and methods

Patients population

Ten MPD patients (6/PV, 2/ET, and 2/IMF), who have been diagnosed to display the JAK2-V617F mutation,
The amplicon length is 843 base pairs (bp). Transcribed with the use of T7 (Fermentas, St. Leon-Rot, Germany) and SP6 (Fermentas) RNA polymerases, at 37°C for 70 min according to the manufacturer’s instructions. We also used Ribolock RNase Inhibitor (Fermentas) in a concentration of 1 U/μL of reaction. The reaction is terminated using equal volume 0.5 mM EDTA and heating at 94°C for 3 min.

2 The hybridization of the opposing RNA strands was following the in vitro transcription. In common practice, sense and antisense strands of patient’s transcripts are mixed in equal volumes and incubated at 94°C for 3 min, while the same procedure is repeated for wild-type transcripts to provide the control sample. For optimum efficiency in the rare cases of high allele burden of V617F mutation sense and antisense strands of patient’s transcripts are additionally cross-hybridized with antisense and sense wild-type strands. The samples are left to cool down over a few minutes to hybridize.

3 After hybridization, the RNA hybrid products are subject to RNase digestion for identification of mutations by cleavage of mismatches. The RNases used are RNase T1 (1/250 dilution, stock solution 100 U/μL; Ambion, Austin, TX, USA), RNase T1 (1/3 dilution, stock solution 1000 U/μL; Fermentas), and a mixture of these (1/500 and 1/6 dilutions, respectively). Samples are incubated with RNases at 37°C for 45 min in digestion buffer (1 mM EDTA pH. 8.0, 10 mM Tris HCl pH. 7.5, 150 mM NaCl, 3 mM CaCl2, 50 μg/mL ethidium bromide). The digestion is terminated using a loading solution consisting of 3 mM NaCl, 10 mM Tris HCl (pH. 7.5), 2 mM EDTA (pH. 8.0), 0.25% bromophenol blue, 10 μg/mL ethidium bromide, and 33% glycerol. Digestion products are subjected to electrophoresis on 1.5% TBE agarose gel.

Verification of NIRCA results by direct sequencing

PCR products were purified by the QIAquick gel extraction kit (Qiagen, West Sussex, UK) and directly sequenced either by using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a Big Dye Terminator DNA sequencing kit (Applied Biosystems) or sequencing service by Macrogen Inc (http://www.macrogen.com/eng/macrogen/macrogen_main.jsp).

Results

Detection of JAK2 mutations into exons 12 and 14 using NIRCA

Non-isotopic RNase cleavage assay assay in all 10 healthy individuals did not yield any DNA fragments (Fig. 1A). Direct sequencing confirmed the NIRCA findings. All 10 patients positive to V617F mutation showed
the same digestion pattern (Fig. 1Bi), demonstrating two distinctive DNA fragments at 593 and 211 bp (Fig. 1Bi arrows) which directly correspond to the position of V617F mutation within the amplicon (T7 and SP6 promoters excluded). The presence of V617F mutation was further verified by direct sequencing (Fig. 1Bii).

Furthermore, four previously analyzed JAK2-V617F negative patients were subjected to NIRCA analysis. Surprisingly, one ET patient demonstrated the same pattern of digestion (Fig. 1Ci) as JAK2-V617F positive patients. NIRCA showed faint digestion products, suggesting lower mutation burden present in the cells of the patient. Direct sequencing verified the presence of V617F mutation in a small percentage of patient’s cells (Fig. 1Ci). Positive digestion pattern was also observed in two idiopathic erythrocytosis patients. However, the pattern was not similar to the one observed in V617F positive patients (Fig. 1Bi, Ci). Digestion yielded two distinctive DNA fragments at 426 and 378 bp (Fig. 1Di) indicating a potential mutation at exon 12. Direct sequencing demonstrated the presence of N542-E543del in patients’ granulocytes (Fig. 1Dii).

The sensitivity of the method was assessed using RNA derived from a patient’s polymorphonuclear cells (PMNs) indicating nearly homozygous V617F allele burden as estimated by sequencing (Fig. 1Bii) and NIRCA after hybridization of patient T7 and SP6 strands, demonstrating digestion fragments barely visible because of the lack of sufficient normal JAK2 allele (Fig. 2B). The patient’s RNA was serially diluted in normal JAK2 RNA in dilutions ranging from 1/2 (50%) up to 1/500 (0.2%). Digestion of mixed RNAs demonstrated digestion fragments up to 1/200 dilution of patient RNA (Fig. 2C–E) thus resulting in sensitivity close to 0.5% for the technique. The threshold of the technique was 5% using PBMCs, as determined by the presence of digestion fragments in dilution 1/20 (data not shown).

Discussion

The diagnosis of the myeloproliferative disorders has been until recently relied on clinical and laboratory data. The discovery of the JAK2-V617F mutation was a breakthrough in the evaluation of these disorders, providing the opportunity for molecular diagnosis, even though the discovery of a JAK2 mutation is not diagnostic for the identification of a particular subgroup of MPD (4–6). The detection of additional mutations in exons 12–15 (8–13) of JAK2 in V617F negative MPD patients raised the need for sensitive routine genetic screening of the entire coding region associated with these mutations, to improve the accuracy in the diagnosis of these diseases.

In this report, we present the application of NIRCA as a first screening method for the simultaneous screening of the coding region from exon 11 to 15 of JAK2 in the neutrophil derived RNA level, which is essential considering the frequency of exons 12 and 14, other than V617F mutation, mutations in V617F negative MPD patients (13). The accuracy of the assay was confirmed by direct sequencing. All 10 MPD patients with
V617F were found positive after NIRCA application, while all 10 healthy individuals were found negative, resulting in high accuracy. Two V617F negative patients (15) with idiopathic erythrocytosis were found to carry the N542-E543 deletion of exon 12. The sensitivity of the method was proven in the case of the correct characterization of a V617F positive ET patient, who was previously found negative probably because of the low neutrophil mutated allelic burden since the previous method (15) did not utilize PMNs. The increased sensitivity achieved through the use of this technique (up to 0.5% sensitivity) could provide a very potent and helpful analytical tool in conjunction with direct sequencing for the identification of mutations present in JAK2, avoiding multiple sequencing to verify somatic mutations at a very low percentage in the patient’s cell population (18, 19). Furthermore, the fact that NIRCA results in specific digestion patterns for specific mutations could provide an analytical method without the need of direct sequencing (18). Moreover, granulocytes from patients with exon 12 JAK2 mutations are mildly involved (12) and as a result a sensitive assay, like NIRCA, together with bone marrow analysis instead of polymorphonuclear cell analysis, could possibly further improve the sensitivity of the method. Additionally, the analysis of JAK2 using NIRCA could be applied for any JAK2 coding region with appropriate primers.

In conclusion, NIRCA in JAK2 cDNA-based analysis is a rapid and low cost technique which could be used in any laboratory without the need of sophisticated equipment (e.g. real time PCR, sequencer etc.) and has been proven to be sensitive in the identification of even low allele burden JAK2 mutations in MPD patients. The technique provides an analytical tool for the screening of the whole exon 11–15 JAK2 coding region while providing high accuracy on negative results. As a result, its application could be used as a rapid screening test for the identification of MPD patients.

References


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