Clinica Chimica Acta 411 (2010) 2097–2100

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Short communication

Rapid detection of JAK2 V617F mutation using high-resolution melting analysis with LightScanner platform

Jun Qian [a], Jiang Lin [b,*], Dong-Ming Yao [b], Qin Chen [a], Gao-Fei Xiao [b], Run-Bi Ji [a], Yun Li [a], Jing Yang [a], Zhen Qian [a]

[a] Department of Hematology, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu 212002, PR China
[b] Laboratory Center, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu 212002, PR China

ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form 13 August 2010
Accepted 13 August 2010
Available online 19 August 2010

Keywords:
JAK2 V617F
Mutation
High-resolution melting analysis
LightScanner
Myeloproliferative neoplasm

ABSTRACT

Background: Detection of the JAK2 mutation has recently been included under the essential diagnostic criteria for myeloproliferative neoplasm (MPN). High-resolution melt (HRM) curve analysis, a nongel-based, automated system, is introduced as a means of mutation scanning without the requirement of any post-PCR handling.
Methods: We studied the sensitivity and reproducibility of LightScanner™ platform in the detection of JAK2 V617F mutation and the availability for diagnostic use in MPN.
Results: The reproducible sensitivity of HRM analysis with LightScanner™ platform was 5% for the detection of JAK2 V617F mutation. In the test of blind screening of 105 samples (48 Ph− MPN and 57 Ph+ chronic myeloid leukemia), the identical judgement was interpreted by two blinded investigators. HRM analysis of all cases was fully concordant with the results of PCR-RFLP and direct sequencing.
Conclusions: The HRM method developed here is an extremely sensitive, accurate and reliable technique and allows high-throughput, fast pre-screening to select for sequencing only those specimens that most likely contain mutant JAK2 V617F allele(s).

1. Introduction

The Janus kinase 2 (JAK2) plays an important role in normal hematopoietic growth factor signaling. Recently, a novel somatic point mutation (c.1849G>T, GTC→TTC) in JAK2 has been observed in several chronic myeloid disorders, most frequently in chronic myeloproliferative neoplasm (MPN), such as polycythemia vera (PV) (65–97%), essential thrombocythemia (ET) (23–57%) and primary myelofibrosis (PMF) (35–57%) [1–8]. This mutation results in substitution of phenylalanine for valine (V617F), both hydrophobic nonpolar amino acids, at position 617 of the JAK2 protein within the JH2 pseudokinase domain [1]. The JAK2 V617F mutation causes constitutive activation of the kinase, with deregulated intracellular signaling that mimics continuous hematopoietic growth factor stimulation. Detection of the JAK2 mutation has recently been included under the essential diagnostic criteria for PV, ET, PMF by the World Health Organization (WHO) diagnostic criteria [9,10].

Several methods have been established to detect JAK2 V617F mutation, including direct DNA sequencing, amplification refractory mutation system (ARMS), pyrosequencing methods, PCR followed by restriction fragment length polymorphism (RFLP) gel electrophoresis, denaturing high-performance liquid chromatography (dHPLC), or probe real-time PCR and DNA melting-curve analysis [1–6,8,11,12]. Each of these methods has its own merits and limitations, including sensitivity, ease of use and instrumentation constraints. Major disadvantages of these assays are that they lack sufficient sensitivity, are rather time-consuming and/or expensive to be used on the large scale for molecular diagnostic purpose.

More recently, high-resolution melt (HRM) curve analysis, a nongel-based, automated system, is introduced as a means of mutation scanning without the requirement of any post-PCR handling [13]. This method is simple and consists of PCR, followed by a short melting step and subsequent analysis. A saturating doubling-stranded DNA (dsDNA) binding dye, such as LCGreen Plus (Idaho Technology, Salt Lake City, USA), is introduced during DNA amplification, which enables differentiation of PCR products based on their dissociation behavior as they are subjected to increasing temperatures [13]. The melting profile of the PCR product depends on its GC content, length, sequence, and heterozygosity. Heterozygous mutations in the DNA sequence will give rise to heteroduplexes that change the shape of the melting-curve when compared to the wild-type melt profile and homozygous mutations will result in the Tm shift compared with wild-type [13–16]. Although several platforms using this new technique have been described to detect JAK2 V617F mutation [17–

* Corresponding author. Laboratory Center, Affiliated People's Hospital of Jiangsu University, Zhenjiang, 212002, PR China. Tel.: +86 511 88915582; fax: +86 511 85236387.
E-mail address: linjiangmail@sina.com (J. Lin).

0009-8981/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2010.08.020

22], we present the first assessment study on the LightScanner™ (Idaho Technology) for diagnostic use in MPN.

2. Materials and methods

2.1. Patient samples and DNA extraction

105 patients were enrolled and bone marrow samples were collected between August 2004 and May 2010. The samples included 3 cases of PV, 40 cases of ET, 5 cases of PMF, and 57 cases of chronic myeloid leukemia (CML). All samples were obtained with informed consent. The bone marrow mononuclear cells were separated by density-gradient centrifugation using Ficoll. Subsequently, the DNAs were extracted using the Genomic DNA Purification Kit (Gentra, USA) according to the manufacturer's instructions.

2.2. Assay design and PCR conditions

Primer design was performed with LightScanner primer design software v1.0 (Idaho Technology, Salt Lake City, Utah). The sequences of primers are as follows: 5′ AAGCAGCAAGTATGATGAGC-3′ (forward) and 5′ AGAAAGGCATTAGAAAG CCTG TA-3′ (reverse). The 61-bp PCR products with a single band were visualized on 2% agarose gels after staining with ethidium bromide. Amplicon melting data were calibrated relative to internal temperature controls, which were composed of complementary oligonucleotides of low (Tm ~ 70.25 °C) and high (Tm ~ 88.85 °C) Tm. The calibrators included in each reaction vessel provided PCR-independent melting signatures. The sequences of calibrators were 5′-ATCGTGATTTCTATAGTT ATCTAAGTAGTTGG-CATTAATAATTTCATTTT-3′and 5′-GCGGTCAGTCGGC CTAGCGGTAGC-CAGCTGCGGCACT GCGTGACGCTCAG-3′ [23]. The internal calibrators were blocked on their 3′-hydroxyl termini with a phosphate group (Genecore, Shanghai, China).

PCR was performed in 25-μL volumes in the presence of 1× PCR buffer (Invitrogen, Merelbeke, Belgium), 0.2 mmol/L of each dNTP, 2.5 mmol/L of MgCl2, 0.4 μmol/l of both forward and reverse primers, 1× LCgreen Plus (Idaho Technology Inc. Salt Lake City, Utah), 1 U Taq polymerase (MBI Fermentas, Canada), and 50 ng genomic DNA. PCR reactions were performed on a 7300 Thermo cycler (Applied Biosystems, Foster City, CA, USA). The temperature cycling protocol consists of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and an extension at 72 °C during 30 s. This PCR reaction is followed by another denaturation step at 94 °C for 30 s, followed by cooling down to 25 °C to facilitate the heteroduplex formation.

2.3. Melting-curve analysis

After PCR, each product was transferred to the LightScanner™ (Idaho Technology Inc. Salt Lake City, Utah), in 96-well plates suitable for HRM analysis and covered with a mineral oil overlay. Plates were heated in the LightScanner from 55 °C up to 90 °C with a ramp rate of 0.10 °C/s. The melting-curve analysis was carried out by the Light-Scanner software package with CALL-IT® software (Idaho Technology Inc. Salt Lake City, Utah). Melting profiles were calibrated by internal oligonucleotide controls, and then normalized, temperature shifted, grouped and displayed as fluorescence-vs-temperature plots or subtractive difference plots (−df/dt vs T).

2.4. JAK2 V617F genotyping by RFLP and sequencing

PCR-RFLP was performed to detect the JAK2 V612F mutation according to the previously described procedure [1]. Subsequently, the PCR products were sequenced directly (Shanghai GeneCore BioTechnologies Co., Ltd., China).

2.5. Study design

DNA samples from 105 patients were included in this study. The JAK2 mutation status was initially defined by PCR-RFLP and DNA sequencing analysis. One investigator with knowledge of the genotypes provided these samples. Two blinded investigators analyzed the 105 samples by HRM analysis and assigned them as wild-type, heterozygous mutant or homozygous mutant, independently.

3. Results

3.1. Sensitivity of HRM analysis in identifying the JAK2 V617F mutation

In order to evaluate the sensitivity of the methodology, the wild-type DNA (G/G) was mixed with that of the homozygous (T/T) mutant

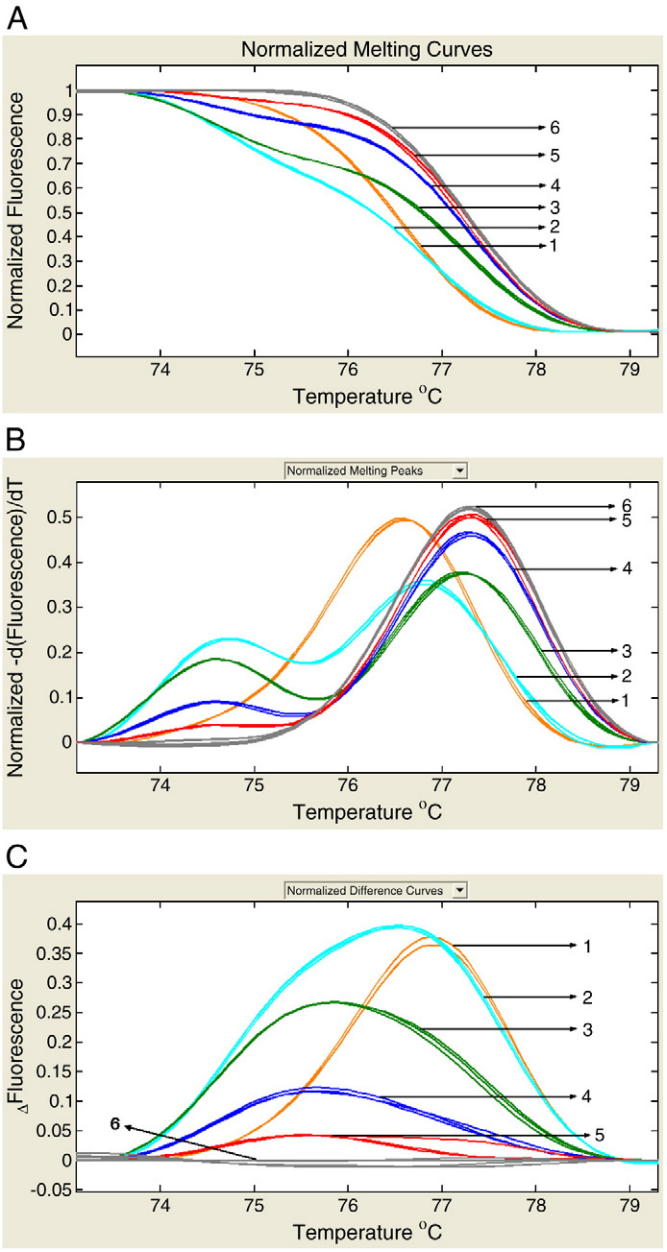

Fig. 1. Results of a dilution series of JAK2 V617F mutant allele diluted in wild-type DNA. 1: 100% mutant; 2: 50% mutant; 3: 25% mutant; 4: 10% mutant; 5: 5% mutant; 6: 1% and 0% mutant. A: normalized melting curves; B: normalized melting peaks; C: normalized difference curves.