Clinical Biochemistry 45 (2012) 345–351

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

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

Four-channel asymmetric Real-Time PCR hybridization probe assay: A rapid pre-screening method for critical BCR-ABL kinase domain mutations

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ARTICLE INFO

Article history:
Received 8 October 2011
Received in revised form 28 December 2011
Accepted 31 December 2011
Available online 12 January 2012

Keywords:
Real-Time
PCR
FRET
Asymmetric
BCR-ABL
Kinase
Imatinib
Dasatinib
Nilotinib
Ponatinib

ABSTRACT

Objectives: Within the laboratory protocols, used for the study of BCR-ABL resistance mutations in chronic myeloid leukemia patients treated with Imatinib, direct sequencing remains the reference method. Since the incidence of patients with a mutation-related loss of response is not very high, it is very useful in the routine laboratory to perform a fast pre-screening method.

Design and methods: With this in mind, we have designed a new technique, based on a single Real-Time FRET-based PCR, followed by a study of melting peaks. This new tool, developed in a LightCycler 2.0, combines four different fluorescence channels for the simultaneous detection, in a single close tube, of critical mutations within the ABL kinase domain.

Results: Assay evaluation performed on 33 samples, previously genotyped by sequentiation, resulted in full concordance of results.

Conclusions: This new methodology detects in a few steps the presence of critical mutations associated to Imatinib resistance.

Introduction

Imatinib resistance is a key challenge in the treatment of patients with chronic myeloid leukemia (CML) [1]. Mutations in the BCR-ABL kinase domain (KD) are one of the typical causes of loss of hematologic or cytogenetic response [2,3]. So far more than 70 different mutations that may affect up to 50 amino acids have been described [4]. Among these, T315I mutation remains one of the biggest challenges due to its total insensitivity to treatment with Imatinib, Dasatinib or Nilotinib; however, the development of new inhibitors such as Ponatinib could be addressing this unsolved problem [5–7]. Therefore the rapid identification of one of the various mutations responsible for first line therapy resistance will allow us to decide to increase the dose of Imatinib, switch to a second generation inhibitor (Dasatinib or Nilotinib) or consider the possibility of undergoing allogenic transplantation or experimental clinical trials (Ponatinib). Nevertheless, the routine diagnosis of BCR-ABL KD mutations associated to Imatinib-resistance remains technically complex. Within the laboratory protocols used in the study of mutations, direct sequencing of ABL KD, with sensitivity up to 25%, remains the reference method [2–4]. However, it is a very *time-consuming* protocol that involves the combination of several laboratory *techniques*. Thus, as the incidence of patients with a mutation-related loss of response is not very high, it is very useful in the routine laboratory practice to perform a fast pre-screening method, from which patients may be selected to move to direct sequencing, saving the unnecessary processing of a large number of samples. From this point of view, we decided to design a new laboratory technique, for the detection in a few steps of the presence of critical mutations within the BCR-ABL KD. The methodology presented in this manuscript is based on a single Real-Time PCR reaction, followed by a study of melting curves. This protocol combines, for the first time, the simultaneous use of 4 pairs of FRET (Fluorescence Resonance Energy Transfer) probes, each emitting at a different wavelength channel (610, 640, 670 and 750 nm). In this context, we decided to apply the methodology used for multiplexed Real-Time PCR reactions, based on the use of asymmetric primer pair concentrations [8–11]. This strategy

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0009-9120/$ – see front matter
doi:10.1016/j.clinbiochem.2011.12.026

significantly increases the fluorescence signal from each channel, allowing the simultaneous use of multiple hybridization probes in a single closed tube. Thus, we target in one PCR reaction, all critical BCR-ABL KD mutations described for Imatinib resistance, from a 625 bp cDNA fragment [4].

Material and methods

Patients, blood collection and RNA isolation

The study was approved by the Scientific Committee of the Hematology Department and was performed retrospectively on a total of 33 bone marrow (BM) and/or peripheral blood (PB) samples collected between 2006 and 2011 from 14 different patients. Median age of patients was 67 (38–94) years, male/female ratio was 50% and disease status was as follows: 78.5% in chronic phase, 7.1% in accelerated phase and 14.2% in blast crisis. In Table 2 are as well described the demographic and baseline patient's characteristics of all the patients/mutations included for the validation of the technique. For RNA extraction, 5 mL of peripheral blood was collected into tubes containing EDTA. RNA was extracted using the RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instructions. Once isolated, the RNA was dissolved in 50 μL of distillated water and quantified in an Ultrospec 4300 pro spectrophotometer. The RNA concentration was adjusted to 100 ng/μL in order to standardize the RNA samples for the PCR reactions. Samples were blinded and all of them were a mix of normal and mutant cases. The cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit, following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

BCR-ABL KD mutation screening method based on specific fluorescently labeled hybridization probes

For the detection of mutations within the KD, associated with critical resistance to Imatinib in CML, we first performed by conventional PCR (Mastercycler gradient, Eppendorf) a first amplification step of the BCR-ABL fragment (1579/1504 bp, depending on b2a3 or b2a2 isoforms) (Table 1). This procedure ensured that the non-rearranged ABL transcript was not analyzed. We next amplified, by Real-Time PCR (LightCycler 2.0), from the first amplification template, a 625 base pair fragment (Fig. 1). The Real-Time PCR included a preheating stage of the mixture at 95 °C for 10 min, followed by 45 cycles of 0 s at 95 °C, 10 s at 60 °C, and 15 s at 72 °C. The sensor and anchor probe sequences used in the Real-Time PCR reaction were designed in the laboratory. The synthesis was performed by TIB MOLBIOL (Berlin, Germany). Both anchor and sensor probes included in the reaction mix were located over or in the vicinity of the mutations (Fig. 1). Anchor probes were labeled at its 5′ end with Red 610, Red 640, Red 670 or Red 705. Adjacent sensor probes were placed 1–3 nucleotides apart from the anchor probes and were labeled with fluorescein at its 3′ end (Fig. 1). Immediately after the Real Time PCR reaction, melting peak analysis was performed on the same LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany) (Fig. 2). The melting assay was based on an initial temperature decrease from 95 °C to 40 °C at a transition temperature rate of 20 °C/s. Then, the temperature was increased at a transition rate of 0.1 °C/s up to 75 °C with continuous fluorescence monitoring. The software provided with the equipment (LightCycler 4.01) gives the melting temperature (Tm) of the sensor/anchor probes. The detection of the nucleotide variation of the gene is based on the fact that the base pair mismatch between the sensor/anchor probe and template causes a decrease in Tm that may be easily detected by a melting peak analysis in the LightCycler 2.0. The reaction mix of both PCRs is described in Table 1.

Table 1
Reaction mix description of each PCR procedure.

	Volume
1° STEP PCR MIX (15 μL) (25 cycles)	
Mix hybridization probes 5× (Master Plus, Hybridization Probes, Roche)	3 μL
H_2O	7 μL
Primer FW (10 μM) 5′-CGCTGACCATCAATAAGG-3′	1.5 μL
Primer RV (10 μM) 5′-GTACTCACAGCCCCACGGA-3′	1.5 μL
cDNA	2 μL
2° STEP Real-Time PCR (15 μL) (50 cycles)	
Mix hybridization probes 5× (Master Plus, Hybridization Probes, Roche)	3 μL
H_2O	4 μL
Primer FW (1 μM) (see Fig. 1 for primer and probe sequences)	0.5 μL
Primer RV (10 μM)	2.5 μL
FLU-A (4 μM)	0.5 μL
610-A (4 μM)	0.5 μL
FLU-B (4 μM)	0.5 μL
640-B (4 μM)	0.5 μL
FLU-D (4 μM)	0.5 μL
670-D (4 μM)	0.5 μL
FLU-C (4 μM)	0.5 μL
705-C (4 μM)	0.5 μL
cDNA (4 μM)	1 μL

Procedure of optimization of the asymmetric multiplex Real-Time PCR

For procedure optimization of the technique we used positive and negative samples for each mutation, already validated by conventional techniques (see Technique validation). Asymmetric amplification, using an excessive amount of one of the primers, allowing the preferential synthesis of the reverse strand complementary to the hybridization probes, causes a significant increase of the fluorescence intensity on the FRET-based Real-Time PCR reaction. The fluorescence increases obtained under these conditions were clearly visualized in the amplification curves as well as in the melting peaks (Fig. 4). Thus, the modification of the primer pair concentration may be considered an important strategy in order to optimize fluorescence signaling coming from a single fluorescence channel [8–11]. Moreover, in the case of a Real-Time PCR, combining 4 different channels for fluorescent emission, the asymmetric strategy becomes an elegant method to overcome the signal loose derived from the use of emission filters. With this in mind we assayed different concentration ratios of the primer pair (1:1/1:10/1:50) (data not shown) with the objective of improving the single channel fluorescence level achieved and the quality of the melting peak for a robust nucleotide genotyping.

Real-Time PCR sensitivity

In order to estimate the sensitivity of the method, based on melting peak analysis, we diluted total RNA from a likely homozygous sample for F317L mutation (almost 100% mutation) with total RNA from a F317L negative sample (Fig. 3). Before diluting mutant and negative RNA samples we adjusted RNA concentration of both samples at 100 ng/μL. The samples selected for the dilution assay shared a closed BCR-ABL/GUS ratio. We obtained samples with 100%, 50%, 25%, 12.5%, and 6.25% of mutation load. As can be observed in Fig. 3, the successive dilutions of the mutant sample decreased the level of the mutated fluorescence melting peak while increasing the normal one.

Technique validation

For technique validation, the 33 samples used for this study were genotyped by reference methods for all the mutations described in this manuscript. The conventional method consisted in a nested PCR followed by DNA template purification from an agarose gel and the performance of DNA fragment sequentiation. We carried out the sequence analysis in ABI 3100 (Big Dye Termination 3.1).