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PATHOLOGIE BIOLOGIE

Pathologie Biologie 57 (2009) 388–391

Original article

Quantitative detection of *bcr-abl* transcripts in chronic myeloid leukemia

Quantification du transcrit *bcr-abl* dans la leucemie myeloide chronique

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Received 22 December 2006; accepted 14 December 2007
Available online 2 April 2008

Abstract

The optimal management of malignant haematological disorders depend on the degree of tumor load reduction after therapy. Chronic myeloid leukemia constitutes a clinical model for molecular detection and therapy surveillance of malignant disease since this entity was the first leukemia shown to be associated with a specific *bcr-abl* fusion gene in the patient's leukemia cells. Molecular monitoring of *bcr-abl* transcript levels by real-time quantitative PCR is increasingly used to assess treatment response in patients with chronic myeloid leukemia (CML). This has become particulary relevant in the era of imatinib therapy when residual levels of leukaemia usually fall below the level of detection by bone marrow cytogenetic analysis. We monitored *bcr-abl* transcript levels by quantitative real time PCR in 50 tunisian patients treated with imatinib for chronic myeloid leukemia in chronic phase for a median of 29 months (3–60) after they started imatinib.

Résumé

Le suivi des hémopathies malignes repose sur l'évaluation de la maladie résiduelle après traitement. La leucémie myéloïde chronique constitue un modèle en oncohématologie. En effet, cette maladie fut le premier processus néoplasique associé à une anomalie génétique acquise et spécifique du clone tumoral qu'est le gène de fusion *bcr-abl*. Le suivi moléculaire du taux de transcrit *bcr-abl* par PCR quantitative est de plus en plus utilisé pour évaluer la réponse des patients au traitement. Cela est devenu particulièrement intéressant à l'ère de l'imatinib lorsque la maladie résiduelle descend en deçà du seuil de détection de la cytogénétique conventionnelle. Nous rapportons le suivi par PCR quantitative de 50 patients Tunisiens atteints de leucémie myéloïde chronique et traités par imatinib avec un suivi médian de 29 mois (3–60) après le début du traitement.

Keywords: Chronic myeloid leukemia; Imatinib; RQ; PCR; Residual disease

Mots clés : Leucémie myéloïde chronique ; Imatinib ; RQ ; PCR ; Maladie résiduelle

1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by acquisition of the philadelphia chromosome (Ph) in leukemic stem cells and their progeny.

The abnormal Ph chromosome is the result of a reciprocal translocation between chromosomes 9 and 22. The major consequence of this translocation is the fusion of the *abl* gene to the *bcr* gene on chromosome 22 [1].

The *bcr-abl* fusion gene encodes a new protein of 190, 210 or 230 Kd, depending on the breakpoint on the *bcr* gene. All these *bcr-abl* fusion proteins have enhanced tyrosine

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0369-8114/$ – see front matter
doi:10.1016/j.patbio.2007.12.010

kinase activity, which is crucial for the development of the disease [2].

Imatinib is a molecularly targeted therapy that inhibits the oncogenic fusion protein *bcr-abl* [3,4].

CML serves as a paradigm for the utility of molecular methods to diagnose malignancy or to monitor patient response to therapy. Several approaches have been introduced that can specifically detect the t (9; 22) or its products, such as fluorescent in situ hybridization, southern blotting, western blotting and RT-PCR.

Since non quantitative RT-PCR analysis gives only limited information, several groups have developed quantitative RT-PCR assays that enable the kinetics of residual -*abl* transcripts to be monitored over time [5,6]

Here, we report the results of serial transcript measurements in 50 tunisian CML patients treated with imatinib.

2. Patients & methods

2.1. Patients and samples

Fifty CML patients (28 male; 22 female) were included in this study. All the patients were Ph positive.the median age at diagnosis was 42 years. All patients received imatinib at a daily oral dose of 400 mg. Prior to imatinib treatment all, patients were in chronic phase.

bcr-abl isoforms were identified by multiplex PCR [2]. Twenty-nine patients expressed b3a2 and 21 patients expressed b2a2.

A total of 118 samples of peripheral blood were analyzed. Approximately two to three samples were investigated from each patient during the course of the disease. The median follow-up was 30 months (6–36).

2.2. RNA extraction and cDNA synthesis

Ten millilitres of EDTA-anticoagulated peripheral blood was collected at diagnosis and every three months after complete cytogenetic remission. The red cells were lysed and residual cells were homogenised in 1 ml trizol.

Total RNA was extracted from 10^6–10^7 peripheral blood cells of patients using the acid guanidinium thiocyanate and phenol chloroform method [2].

After each extraction, integrity of RNA was assessed by migration on a BET agarose gel; Reverse transcription was performed on 1 µg of total RNA. cDNA synthesis was performed according to the manufacturer's instructions using random hexamer priming and MMLV reverse transcriptase.

2.3. Quantitation of bcr-abl transcripts

bcr-abl and *abl* transcripts were quantified using a commercially available fusion Quant kit (IPSOGEN) developed according to the EAC network protocol [7].

cDNA was amplified by 50 cycles of Q-PCR using the ABI 7700 sequence detection system (applied bisystems, fostercity, CA, USA) and taqMAN® Universal PCR Master Mix in accordance with the manufacturer's instructions in a final reaction volume of 25 µl.

The *bcr-abl* and *abl* probes were dual labeled with FAM and TAMRA; the probe and primer concentrations for *abl* mRNA quantification were 200 and 300 nM, respectively.

2.4. Principle of quantitation using taqMAN® probes (Fig. 1)

RQ-PCR analysis using taqMAN® probes exploits the $5' \rightarrow 3'$ exonuclease activity of the thermus aquaticus (taq) polymerase to detect and quantify specific PCR products as the reaction proceeds.

The taqMAN® probe is positioned within the target sequence and is conjugated with a reporter fluorchrome (FAM) as well as a Quencher fluorochrome (TAMRA). As long as the two fluorochromes are in each other's close vicinity, that is, as long as the probe is intact, the fluorescence emitted by the reporter fluorochrome will be absorbed by the Quencher fluorochrome. However upon amplification of the target sequence, the hydrolysis probe is initially displaced from the DNA strand by the taq polymerase and subsequently hydrolysed by the $5' \rightarrow 3'$ exonuclease activity of the taq polymerase. This results in the separation of the reporter and Quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle, this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

A CCD camera measures the target-specific fluorescence emission spectrum from 500 to 650 nm in real time during each elongation step and data are integrated by the sequence detector softwareV1.6.

The threshold cycle (Ct) is defined as the fractionnal cycle where the fluorescence intensity of a sample reached ten times the standard deviation of the baseline. The Ct is invertionally proportionnal to the initial number of copies, thus, if the starting copy number is important, the specific signal is detected early and the Ct value is low.

Theroretically the Ct value of a nondiluted and two fold diluted sample should differ by one, whereas the difference between a nondiluted and 10-fold diluted sample should be –3.3.

A standard curve is generated using serial dilutions ranging from 10^5 to 10 copies of linearised plasmid containing the *bcr-abl* insert. The *bcr-abl* and *abl* copy numbers were calculated by comparison with the standard curve the *bcr-abl* copy number in the sample is read off the standard curve which is generated for each assay.

3. Results

A total of 118 samples of peripheral blood were included in this study.

In order to correct variations linked to differences in the amount of RNA taken for the reaction or to different levels of inhibition during RT or PCR we normalized the