

ORIGINAL ARTICLE

Real-time quantification for BCR-ABL transcripts in chronic myeloid leukaemia patients in UKMMC, Malaysia

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Abstract

Molecular pathogenesis of chronic myeloid leukemia (CML) is well established and molecular monitoring for patients with CML has become an important practice in the management of patients on imatinib therapy. In the present study, we report the use of RQ-PCR method for detection of BCR-ABL fusion gene for our CML cases. We performed a two-step RQ-PCR on bone marrow aspirates or peripheral blood of 37 CML patients. Quantitative expression of BCR-ABL fusion gene was carried out relative to the expression of a housekeeping gene as endogenous control to compensate for uneven cell numbers, RNA quality, or variations in reverse transcription efficiencies. Twenty-four of these patients were pre-treated with hydroxyurea or alpha interferon prior to the imatinib therapy. Their BCR-ABL fusion gene levels were monitored for 18 months. All samples processed were evaluable. The PCR amplification efficiency of the ABL gene is 90.5% (0.2158) and the BCR-ABL gene, 93.4% (0.1573).

Keywords: CML, BCR-ABL, RQ-PCR

INTRODUCTION

Chronic myeloid leukaemia (CML) is a malignant clonal disorder of haemopoietic stem cells, characterised by reciprocal translocation between chromosome 9 and chromosome 22; t(9;22) (q34;q11) which results in the formation of Philadelphia (Ph¹) chromosome.^{1,2} The molecular consequence of this translocation event is the BCR-ABL fusion gene. The chimeric gene encodes a fusion protein with a deregulated kinase activity, that is central to the pathogenesis of CML.³⁻⁶ The constitutively active tyrosine kinase activity, essential to the proliferative advantage of the neoplastic clone and for the progression of disease,⁷⁻⁸ has been the focus of many studies to find a selective inhibitor of such activity, of which imatinib, has been proven to be successful.^{9,10} Results from randomised trials have indicated impressive clinical responses and imatinib is currently the treatment of choice for all phases of CML.¹¹⁻¹⁶

The methods for diagnosis and monitoring response to treatment for CML patients have evolved considerably in recent years. The genetic

defect can be detected at the level of chromosome, fusion gene, chimeric RNA or altered structure of the protein and thus several approaches have been introduced such as fluorescent *in situ* hybridisation (FISH), reverse transcriptase PCR (RT-PCR), Southern blot analysis or Western blot analysis. Quantitative real-time RT-PCR (RQ-PCR), a more sensitive technique to measure BCR-ABL transcript levels, is now the standard method for monitoring the response to treatment of CML patients.¹⁷⁻¹⁹ Here, we report our analysis of BCR-ABL-transcripts in CML cases in UKMMC by real-time PCR.

MATERIALS AND METHODS

Patients, samples and RNA extraction

In this study, quantification of BCR-ABL transcripts by RQ-PCR was done on 37 cases of CML. Samples were taken from bone marrow aspirate and peripheral blood, with written informed consent. Total RNA was isolated using RiboPure TM Blood Kit (Ambion, USA), according to the manufacturer's guidelines. The RNA concentration and purity were determined

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spectrophotometrically at 260 and 280 nm and RNA samples were stored at - 80°C until use.

cDNA preparation

1.0 µg RNA was initially incubated for 10 minutes at 70°C and was reversed transcribed into cDNA in a 20µl reaction mixture (Ipsogen France) containing 25 µM random hexamers, 100 units of Superscript II reverse transcriptase, 20 units RNase inhibitor, 4.0 µl of 5x first strand buffer, 2.0 µl of 0.1M DTT, 2.0 µl of 10mM each of dNTP and 2.0 µl of 50 mM MgCl₂ and incubated according to the manufacturer's protocol.

Experimental Design for RQ-PCR analysis of BCR-ABL transcripts

BCR-ABL and ABL transcripts were quantified using a commercially available FusionQuant Kit (Ipsogen, France) developed according to the EAC network protocol.²⁰ 100 ng of the generated cDNA was amplified in a final volume of 25 µl PCR reaction, in accordance with the manufacturer's protocol for 50 cycles of RQ-PCR using ABI Prism 7000 Sequence Detection System (SDS) (Applied Biosystem, USA). Each sample was done in duplicates. The incubation was at 95°C for 15 minutes, 94°C for 15 seconds and 60°C for 60 seconds. The specific primers (ABL and BCR/ABL) were labeled with internal double dye probe, FAM-TAMRA. PCR amplification efficiency for ABL (control gene)

and BCR/ABL (fusion gene) were determined by serial dilutions of plasmid DNA for BCR/ABL gene (10¹ to 10⁵) and ABL gene (10³ to 10⁵). The Ct cycle was plotted against function of log [10] concentration of templates in order to generate a standard curve. The slope of the trend line will be a function of the PCR efficiency by using the equation of PCR efficiency = $\{10^{(1/-\text{slope})}\}-1$. PCR efficiencies of both genes should be similar and the PCR amplification efficiency should be above of 90%. (Figures 1 to 4).

RESULTS

Real-time PCR analysis of BCR/ABL

The PCR amplification efficiency of the ABL gene was 90.5% (±0.2158) and the BCR-ABL gene, 93.4% (±0.1573). The slope for ABL gene was -3.599762 and that for BCR-ABL was -3.457709 (Figure 4). An ideal slope should be -3.32 for 100% efficiency. Fusion gene and control gene correlated well with R² = 0.99. We were able to detect the BCR-ABL transcripts in all the patients' samples and their respective follow-up samples.

RQ-PCR results

There was a total of 37 cases of CML: 20 males and 17 females. They were treated with imatinib at a daily dose of 400 mg orally. Ethnically, 20 were Malay, 12 Chinese and 5 Indian. The median age of diagnosis was

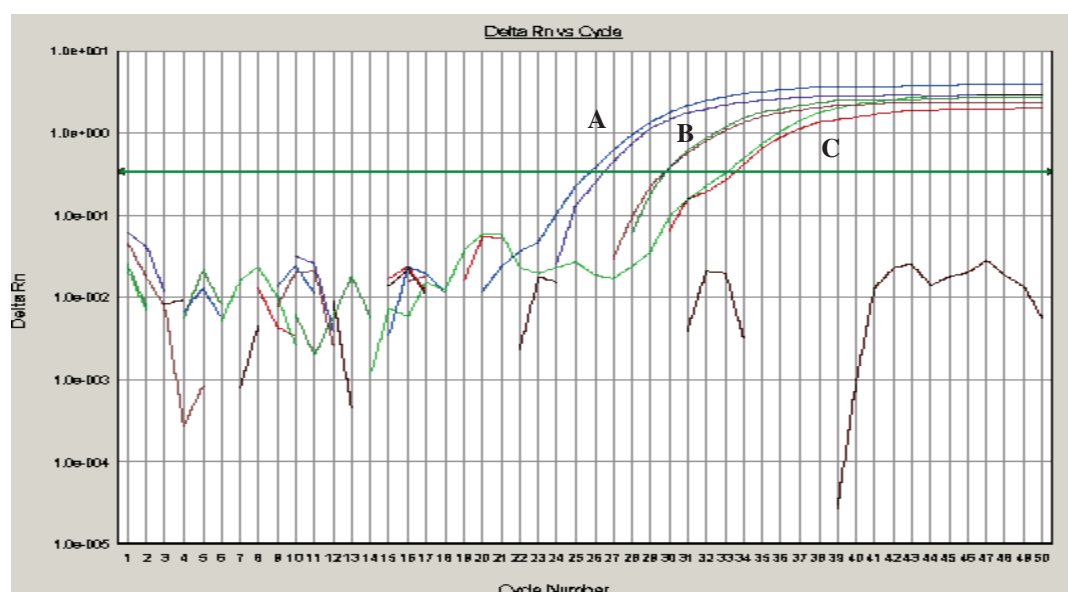


FIG. 1: Amplification plot for ABL (endogenous control): A:1 x 10⁵ copies of ABL genes, B: 1x 10⁴ copies of ABL genes, C:1x10³ copies of ABL genes.

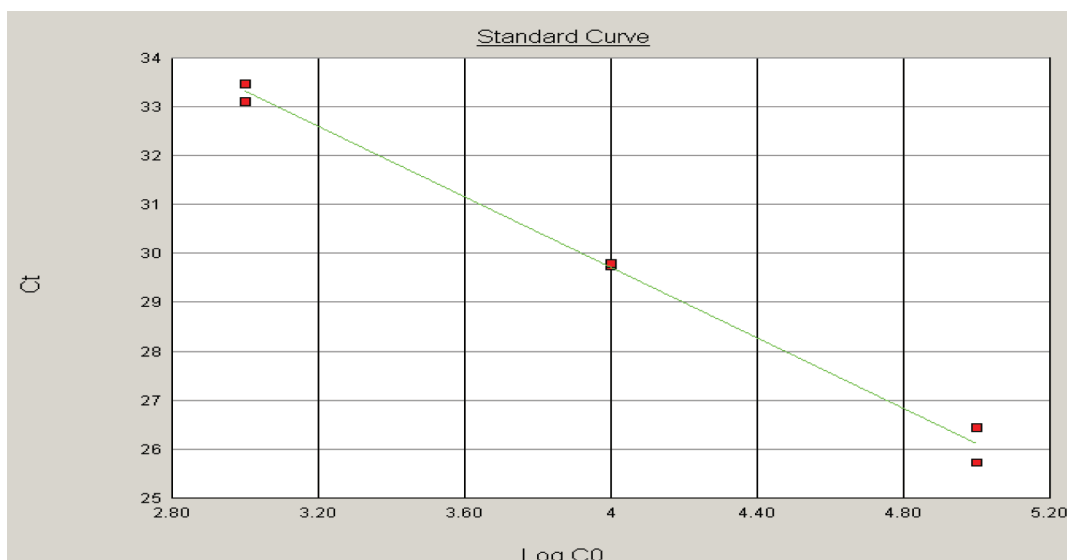


FIG. 2: Std curve plot for ABL gene. (Detector: abl-ip, Slope: -3.599762, Intercept: 44.112000, R2: 0.993673)

44 years. The haematological findings of the patients were consistent with CML in chronic phase. All patients expressed b3a2 fusion gene by conventional RT-PCR. RQ-PCR analyses prior to the imatinib therapy were performed in all cases. Only 13 out of 37 CML cases had their RQ-PCR analyses performed at the time of their diagnosis. They had not received other chemotherapy before and were only treated with imatinib 400 mg daily per oral following their

diagnosis. In this group of new CML cases, all except three cases completed 12 months of imatinib therapy. All cases demonstrated a downward trend of BCR-ABL transcript levels; however only 2 cases achieved major molecular response state. One case showed a log increase at 6-month follow-up. However this patient showed a 2-log reduction after the twelfth month therapy.

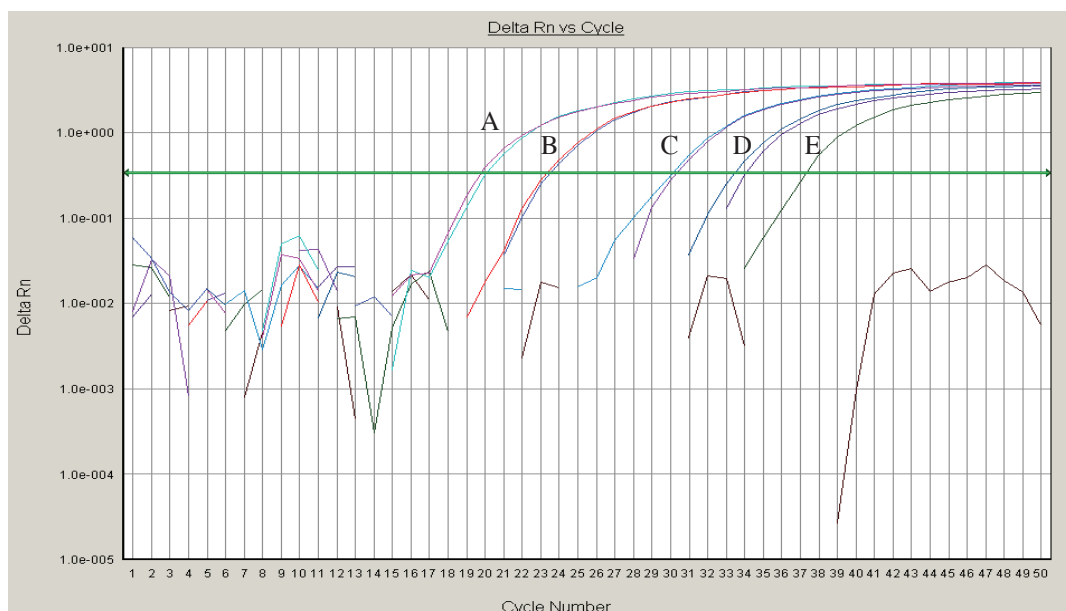


FIG. 3: Amplification plot for BCR-ABL m-bcr fusion gene (target gene).

A- 1×10^6 copies of BCR-ABL m-bcr genes, B- 1×10^5 copies of BCR-ABL m-bcr genes, C- 1×10^3 copies of BCR-ABL m-bcr genes, D- 1×10^2 copies of BCR-ABL m-bcr genes
E- 1×10^1 copies of BCR-ABL m-bcr genes

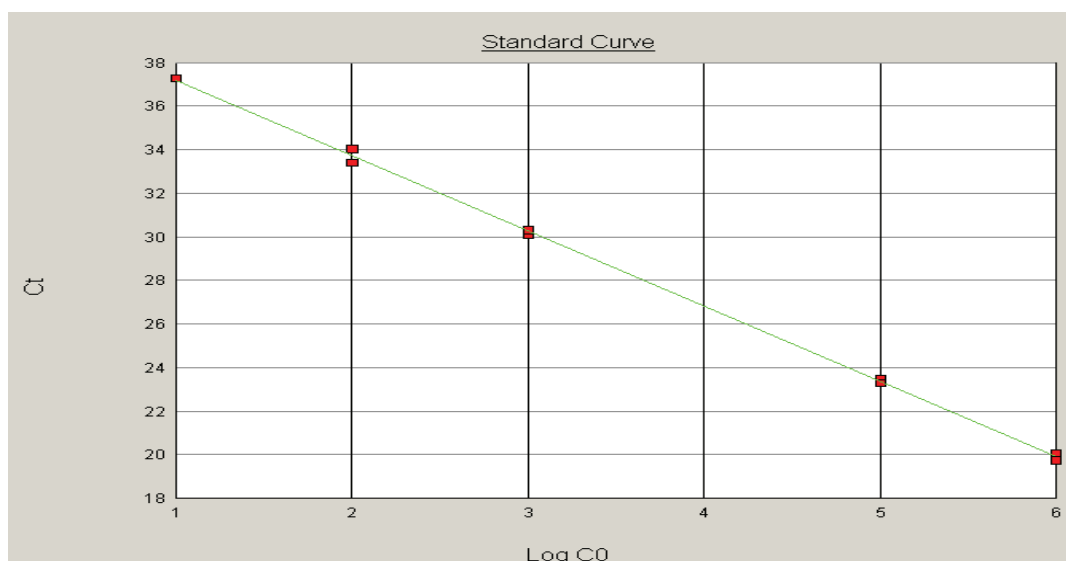


FIG. 4: Standard curve plot for BCR-ABL m-bcr gene.
(Detector: BCR/ABL, Slope: -3.457709, Intercept: 40.642620, R2: 0.999301)

The other 24 cases of CML were also in chronic phase. They had been treated with either hydroxyurea or alpha-interferon prior to the imatinib therapy. Serial monitoring were done for all the 24 cases for a duration of 18 months, 17 of them showed a decreasing levels of the transcripts, while the rest showed an increasing trend (Figure 5).

DISCUSSION

Significant improvements have been made

with respect to the methods for evaluating the therapeutic efficacy on the molecular level in CML. RQ-PCR is a relatively simple, highly sensitive and reproducible method. Quantification of BCR-ABL transcripts by RQ-PCR is also fast as the result could be obtained within a day and no post-PCR processing is needed, thus reducing the risk of contamination.

The discovery of tyrosine kinase inhibitors such as imatinib has revolutionised the management of CML. Detection and monitoring of BCR-ABL transcripts have become an

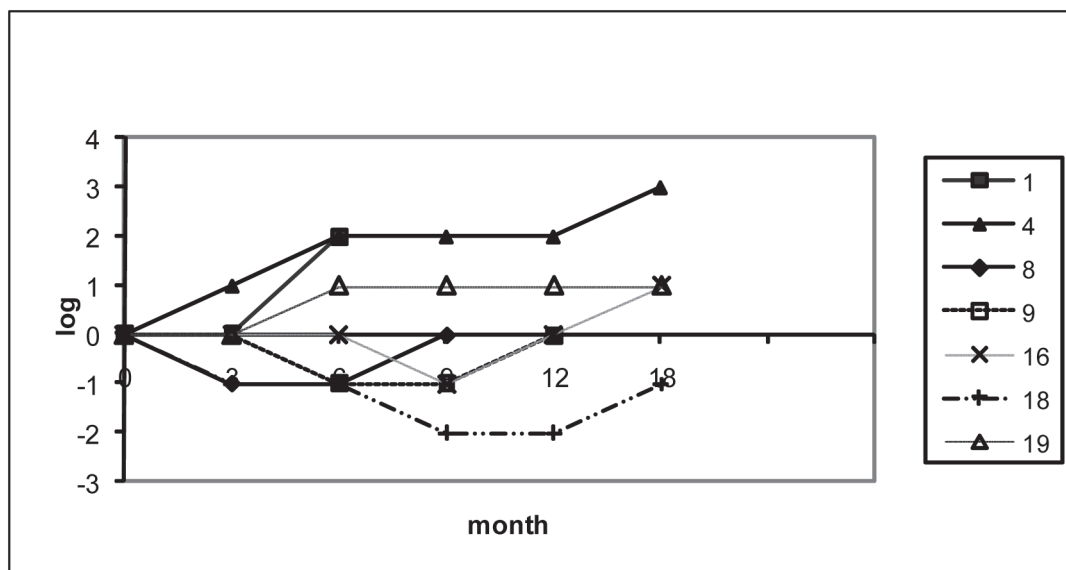


FIG. 5: The plots of 7 CML cases (log increase/decrease from a baseline) at specific time points during therapy with imatinib showing an increasing quantification of transcript.

integral part of management of CML. RQ-PCR analysis used in monitoring has been helpful in determining minimal residual disease or detecting an increasing leukaemia burden. Serial monitoring could improve risk stratification and enable early therapeutic intervention. In patients with sub-optimal response to imatinib, increasing the dose of imatinib from 400 mg daily to 600 – 800 mg daily or scheduling them for allogeneic haemopoietic stem cell transplant could be considered.

Blood samples are easier to collect on a regular basis. The use of peripheral blood enables a more frequent monitoring with a reduction in the frequency of invasive bone marrow aspirates. We have been using RQ-PCR technique to diagnose and monitor the CML cases. Peripheral blood specimens were collected in EDTA anticoagulant in our series of follow-up analysis of CML cases by RQ-PCR.

The onset of ABL mutations has been the most frequent identified mechanism responsible for emergence of resistance, especially in the advanced phases. Resistance was defined according to EAC programme guidelines as evidenced by any haematological, cytogenetic or molecular progression (i.e. increasing BCR-ABL: ABL ratio ≥ 2 logs) of CML in a previously imatinib-responsive patient. This underscores the importance of molecular monitoring of the disease.

In our series of 24 cases who had received either hydroxyurea or interferon showed two patterns of responses on imatinib therapy. The majority showed a downward trend of decreasing quantity while seven of them showed slowly increasing values. Previous studies had reported that increasing transcript quantification was associated with the emergence of mutations which were resistant to imatinib. Observation in these seven patients constitutes a basis for further investigation for mutation analysis in these patients.

In our hand, RQ-PCR is a fast and effective technique. It can replace conventional RT-PCR as a first-line method of molecular diagnosis of CML and it is also useful in monitoring minimal residual disease and genetic recurrence in patients known to harbour this translocation.

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