ORIGINAL ARTICLE

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

FL WONG *BSc,PhD*, NH HAMIDAH *MBBCh, DMedSc*, AA HAWA *BSc*, AN NURUL *BSc*, CF LEONG *MPath,FRCPA*, *SAW FADILAH *FRCPE, PhD* and O AINOON *MBBS, DMedSc*

Haematology Unit, Departments of Pathology and *Medicine, Universiti Kebangsaan Malaysia Medical Centre, Universiti Kebangsaan Malaysia, Kuala Lumpur.

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 (Ph[1]) 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

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 $MgCl_2$ 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 (10^1 to10^5) and ABL gene (10^3 to 10^5). 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 = $\{10^{(1/-slope)}\}$-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
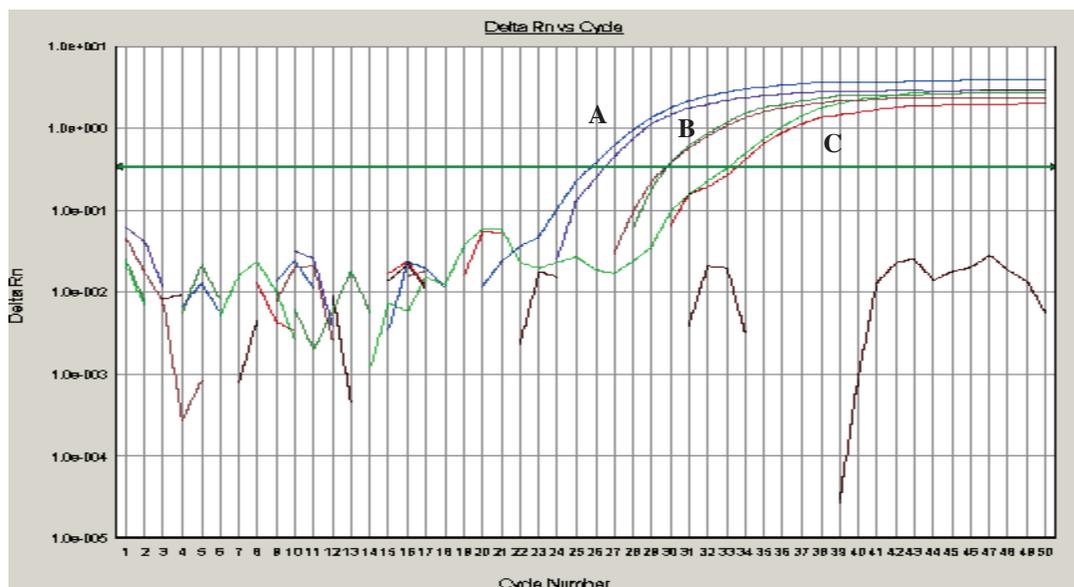

FIG. 1:　Amplification plot for ABL (endogenous control):　A:1 x 10^5 copies of ABL genes, B: 1x 10^4 copies of ABL genes, C:1x10^3 copies of ABL genes.
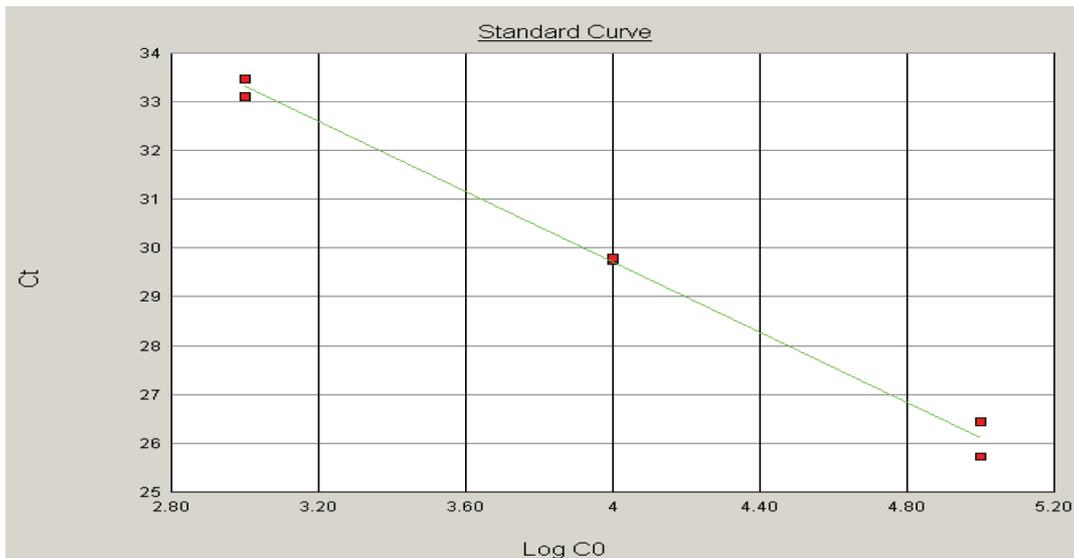
FIG. 2: Std curve plot for ABL gene. (Detector: abl-ip, Slope: -3.599762, Intercept: 44.112000, R2: 0.993673)

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 twelveth month therapy.
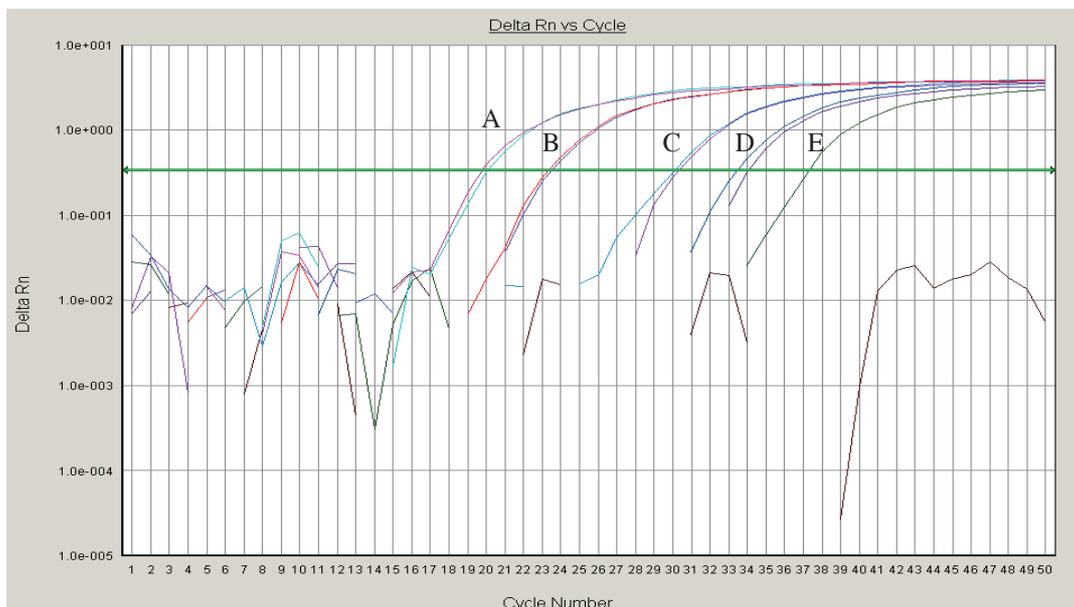

FIG. 3: Amplification plot for BCR-ABL m-bcr fusion gene (target gene).
A-1×10^6 copies of BCR-ABL m-bcr genes, B-1×10^5 copies of BCR-ABL m-bcr genes, C-1×10^3 copies of BCR-ABL m-bcr genes, D-1×10^2 copies of BCR-ABL m-bcr genes
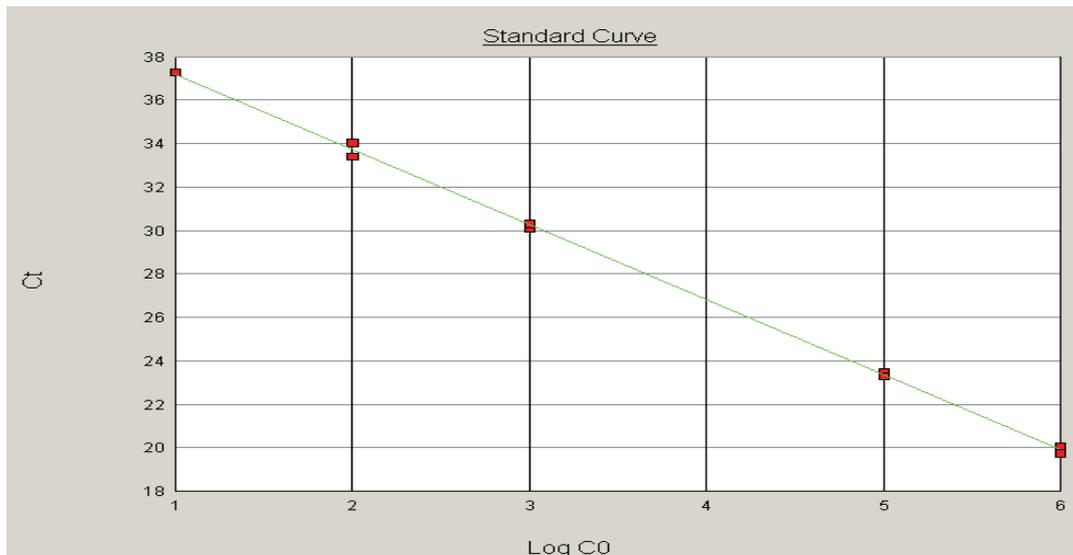E-1×10^1 copies of BCR-ABL m-bcr genes

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 revolutionalised the management of CML. Detection and monitoring of BCR-ABL transcripts have become an
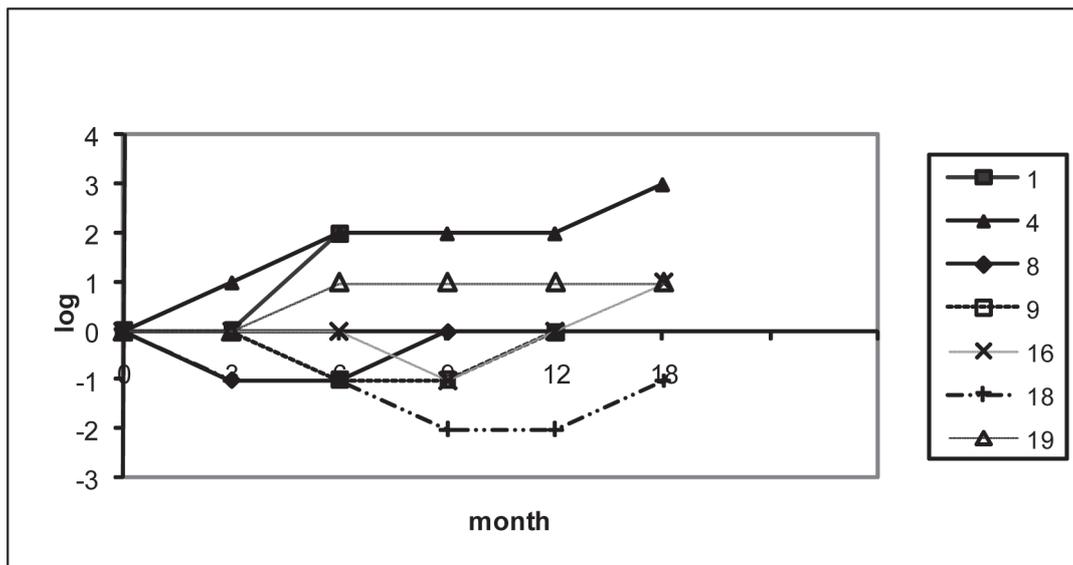

FIG. 5: The plots of 7 CML cases (log increase/decrease from a baseline) at specific time points during therapy with imatinib showing an increasing quantification of transcript.

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.

ACKNOWLEDGEMENTS

REFERENCES

1. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulcytic leukaemia. Science 1960;132:1497-9.
2. Rowley JD. A new consistent chromosomal abnormality in chronic myeloid leukaemia identified by Quinacrine fluorescence and Giemsa staining. Nature 1973;243:290-3.
3. Groffen J, Stephenson JR, Heisterkamp N, de Klien A, Bartram CR, Grosveld G. Philadelphia chromosome breakpoints are clustered within a limited region, bcr , on chromosome 22. Cell 1984;36:93-9.
4. Shtivelman E, Lifshitz B, Gale RP, Cannani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature 1985;315:550-4.
5. Davis RL, Konopa JB, Witte ON. Activation of the c-abl oncogene by viral transduction generates c-abl proteins with similar in vitro kinase properties. Moll Cell Biol 1985;5:204 -13
6. Daley CQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukaemia in mice by P210BCR/ABL gene of the Philadelphia chromosome. Science 1990; 247:824-30.
7. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 1990;247:1079-82.
8. Druker BJ, Tamura S, Buchdunger E, *et al*. Effects of a selective inhibitor of Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996;2:561-6.
9. Salesse S, Catherine CM. BCR/ABL: from molecular mechanisms of leukaemia induction to treatment of chronic myelogenous leukaemia. Oncogene 2002;21:8547-59.
10. Druker BJ, Laydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukaemia. J Clin Invest 2000;105:3-7.
11. Druker BJ, Talpaz M, Resta DJ, *et al*. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukaemia. N Engl J Med. 2001;344:1031-7.
12. Sawyers CL, Hochhaus A, Feldman E, *et al*. Imatinib induces haematologic and cytogenetic responses in patients with chronic myelogenous leukaemia in myeloid blast crisis: results of a phase II study. Blood 2002; 99:3530-9.
13. Kantarjian H, Sawyers C, Hochhaus A, *et al*. Haematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukaemia. N Engl J Med 2002;346:645-52.
14. O Brien SG, Guilhot F, Larson RA, *et al*. Imatinib compares with interferon and low dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukaemia. N Engl J Med 2003;348:994-1004.
15. Druker BJ, Guilhot F, O'Brien SG, *et al*. Five-year follow-up of patients receiving imatinib for chronic myeloid leukaemia. N Engl J Med 2006;355:2408-17.
16. Hochhaus A, Druker B, Sawyers C, *et al*. Favourable long-term follow-up results over 6 years for

response, survival and safety with imatinib mesylate therapy in chronic-phase chronic myeloid leukaemia after failure of interferon-alpha treatment. Blood 2008;111:1039-43.

17. Paschka P, Muller MC, Merx K, *et al*. Molecular monitoring of response to imatinib (Glivec) in CML patients pretreated with interferon alpha. Low levels of residual disease are associated with remission. Leukemia 2003;17: 1687-94.

18. Marin D, Kaeda J, Szydlo R, *et al*. Monitoring patients in complete cytogenetic remission after treatment of CML in chronic phase with imatinib: patterns of residual leukaemia and prognostic factors for cytogenetic relapse. Leukaemia 2005;19:507-12.

19. Martinelli G, Iacobucci I, Soverini S, *et al*. Monitoring minimal residual disease and controlling drug resistance in chronic myeloid leukaemia patients in treatment with imatinib, as a guide to clinical management. Haematol Oncol 2006;24:196-204.

20. Gabert J, Beillard E, van der Velden VH, *et al*. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003; 17:2318-57.