Original Research Article

Molecular Quantification of BCR/ABL Ratio in CML and usefulness in Prognosis and Evolution of Disease

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Abstract

Backround: Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm that originates in abnormal pluripotent bone marrow stem cell and is associated with the BCR ABL1 fusion gene located in the Philadelphia chromosome. *Objective:* Molecular diagnosis is done by detecting BCR-ABL fusion transcripts which is also helpful in the identification of various molecular subtypes on the basis of breakpoint cluster regions involved in the translocation. *Materials & Methods:* This was a data based study & a total of 90 patients were enrolled in the study. The molecular diagnosis was done on Real Time PCR in a stepwise manner using commercial kits. Firstly RNA was extracted from blood kits, then cDNA was synthesized which was subjected to quantitative PCR & the BCR-ABL/ABL ratio was calculated. *Results:* Out of the 90 patients who reported with mild to moderate leucocytosis Philadelphia chromosome was not detected in 14 patients, 18 patients were with negative BCR-ABL/ABL ratio and 58 patients were found to be positive with mean value of 36.17±7.43 of the ratio which decreased with the treatment. *Conclusion:* Molecular detection of the BCR-ABL/ABL ratio is an important tool for assessing response to therapy and in developing more effective therapeutic modalities.

Keywords: Polymerase chain reaction, molecular diagnosis, Philadelphia, Leukemia, myelogenous, BCR-ABL positive.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm that originates in an abnormal pluripotent bone marrow stem cell involving the It involves the myeloid, erythroid, megakaryocytic, B and sometime T-lymphoid but not the marrow fibroblasts [1].

In FAB classification, CML are classified under chronic myleoproliferative disease [2]. But according to classification by WHO only the Ph+:t(9;22)(qq34;q11), bcr-abl cases are called CML and the Ph-ve cases are called atypical CML and belong to the newly created myleodysplastic /myleoproliferative group [3].

CML can occur at any age but the median age at the diagnosis is 5th decade with slight male predominance. The natural history of untreated CML is bi or triphasic: an initial indolent chronic phase (CP), lasting for several months to several years with increased cellularity of the bone marrow followed by an accelerated phase (AP), having increasing WBC ($>10 \times 10^9$/L) chromosome changes in the leukemic cells and a blast crisis phase(BP) with blasts equal to or greater than 20% of peripheral blood [4].

Most patients are diagnosed in the CP phase of the disease, which usually has an insidious onset, Nearly 20-40% of patients are asymptomatic and are diagnosed when a white blood cell count (WBC) performed at the time of routine medical examination is found to be abnormal. Common features at presentation include fatigue, weight loss, night sweats, splenomegaly and anaemia. Atypical presentation may include marked thrombocytosis unaccompanied by significantly elevated WBC count as well as the initial presentation in BP without a previously detectable CP [5]. As the patient enters the AP the activation of tyrosine kinase oncoprotein occurs disrupting the normal regulation of haematopoiesis, causing increased cell proliferation loss of differentiation and cell survival [6].

The diagnosis of CML can be made even in the presence of mild leucocytosis provided BCR-ABL positivity is documented as it is consistently associated with the BCR/ABL fusion gene located in Philadelphia chromosome (Ph`).This chromosome is the hallmark cytogenetic abnormality of CML and results from reciprocal translocation between chromosome 9 and 22. This translocation transposes the C-abl (abelson leukemia virus) proto-oncogene from its normal location on chromosome 9, to a new position on chromosome 22, near to C-bcr (break point region cluster). This genetic alteration results in the formation of a chimeric protein, bcr-abl. It produces an abnormal 8.5 Kb RNA that encodes for 210 KD fusion protein. It is demonstrated that p210 acts by releasing controls on stem cell proliferation or by blocking programmed cell death in ways that lead to CML [7].

This was a data based study and a total of 90 patients were enrolled in the study. Firstly the quantification of BCR-ABL/ABL ratio by RT-PCR was done as a routine test and the cytogenetic response for event free and overall survival was seen.

We also aimed at detecting BCR-ABL fusion transcripts and compared it with classical PCR to further detect residual disease.

MATERIALS AND METHODS
CML was provisionally diagnosed on the basis of peripheral blood smear and bone marrow findings of the patients attending the Hematology Clinic at Pt. BDS PGIMS Rohtak. The 90 patients who reported with mild to moderate leucocytosis where subjected to quantitative RT PCR.

The molecular diagnosis was done on Real time PCR in a step wise manner using commercial kits. Firstly RNA was extracted from blood kits then a cDNA was synthesised which was subjected to real time PCR.

Total leukocyte RNA was extracted from 6 mL of peripheral blood in tubes with EDTA. It was ensured that the samples were coded and labelled properly. Samples were kept at room temperature and processed within 24 h after collection to avoid RNA degradation. Red cells were lysed and residual cells were homogenized in 1 mL of RNA stabilization solution and stored at -80ºC. RNA was extracted according to manufacturer's instructions. (QiAmp RNA blood mini kit). RNA integrity was assessed by electrophoresis in agarose gel.

c-DNA was synthesized by using the commercial kits from Thermoscientific in a two step strand synthesis using the RNA, buffer solution, dNTps and reverse transcriptase enzyme. This mixture is incubated at 42ºC for 1 hour followed by final incubation at 70ºC for 10 minutes to cause final extension.

Quantitative real-time PCR- Rapid quantification of the target sequence during the extensive phase of PCR was to be done. The c -DNA was amplified by using the specific primers and subjected to 35 cycles of RT-PCR using the TAQMAN Universal Master Mix in accordance with the manufacturer's instructions (Qiagen BCR-ABLMBCR kit) in a final reaction volume of 15 µL. ABL was used as the control gene. The primers used were as followed:
- Forward :5'-GATACGAAGGGAGGG TGTACCA-3';
- Reverse : 5'-CTCGGCCAGGGTGTTGAA-3'

A standard curve was generated using serial dilutions of a linearized plasmid containing a BCR-ABL insert. Duplicates were performed for standard curves and patients. The results were reported as a BCR-ABL/ABL ratio (%). The absolute copy no. of a target sequence (BCR-ABL) or a control gene (ABL) can be calculated using a calibration curve prepared from a set of BCR-ABL RNA standards.

RESULTS
The Total leucocytes count in chronic myeloid leukemia patients at the time of presentation is as follows showing leucocytosis (Table-1).

Table-1:

TLC at presentation (per cubic mm)	Male	Female	Total no. of patients
≤ 1 lakh	45	11	56
1 – 2 lakh	18	16	34
Total	63	27	90

The bone marrow reports as available with the patients also showed hypercellular bone marrow with greatly increased Myeloid to erythroid ratio.

Table-2:

Patients (n=90)	Philadelphia Chromosome	BCR-ABL/ABL Ratio
14	Negative	-
18	Positive	≤ 0 .1
58	Positive	36.17±7.43

When subjected to real time quantitative PCR out of 90 patients 14 patients showed negative Philadelphia with undetectable BCR-ABL transcripts. BCR-ABL/ABL ratio was found to be ≤ 0.1 in 18 patients while 58 patients reported with BCR-ABL/ABL ratio as 36.17±7.43 (Table-2).

The 58 patients were given standard imatinib therapy for 12 weeks with the result that only 11 patients showed molecular relapse and the remaining 47 patients showed decreased BCR-ABL/ABL ratio after 12 weeks of standard imatinib therapy (Table-3). These 47 patients also reported with decrease in the WBC count by 5-7 log value than the initial one as per the hematological reports.

Table-3:

BCR-ABL/ABL Ratio	Ratio after 12 weeks of treatment	p value
36.17 ±7.43	12.54±3.75	<0.05

Real time quantitative RT-PCR result showed that (26 patients) rapid decrease in BCR-ABL/ABL ratio was observed and it was followed by complete hematological and cytogenetic remission often defined as major molecular response (MMR) where as a very slow decrease (21 patient) was associated with lack of clinical response.

DISCUSSION

In all these cases quantitative RT-PCR was well corelated to clinical evolution and appeared to give earlier prediction of clinical outcome. In this study we again monitored CML patients treated with imatinib in the chronic phase by RT-PCR. The drug acts by blocking the binding of ATP to bcr-abl tyrosine kinase thus inhibiting kinase activity, which in turn inhibits proliferation and induces apoptosis in bcr-abl positive cell lines [8]. Samples were collected three months after starting imatinib therapy and our results showed that Imatinib induces rapid and complete hematological response in almost all Ph$^+$ patients with CML which is in accordance with the other studies [9]. Also a positive correlation between cytogenetic and molecular response was seen as described in other studies [10].

The MMR is said to be achieved when BCR-ABL is undetected by quantitative RT-PCR. It indicates nonquantifiable and nondetectable BCR-ABL transcripts i.e. BCR-ABL /ABL ≤ 0.10. Also there is three log reductions of BCR-ABL /ABL ratio. Loss of molecular response indicates quantifiable and detectable BCR-ABL transcripts i.e. BCR-ABL /ABL ≥ 0.10. Also there is no three log reductions of BCR-ABL /ABL ratio [11]. The loss of major cytogenetic response (MCR) is 0 to 35% Ph positive, or complete cytogenetic response (CCR, Ph negative) [12, 13].

A significant proportion of patients achieved CCR and MMR (45% and 36%, respectively). In general, after 12 months of imatinib therapy. It was also observed that 18 % of the patients were detected of having the residual disease. Among patients with CCR after 12 months of imatinib treatment, those with MMR have a significantly improved progression-free survival compared to those without MMR as seen in other studies also [14]. Our analysis was retrospective and various physicians treated the patients on an individual basis. No controlled trials of therapeutic options to overcome or prevent the resistance for the patients with various disease phases were undertaken. Real-time quantitative PCR is a useful tool for monitoring chronic myeloid leukemia disease progression and to detect any residual disease but it is still doubtful can it replace bone marrow cytogenetics for diagnosis or not [15, 16].

CONCLUSION

Real time PCR may show better correlation with clinical and cytogenetic evolution than conventional technique and of BCR-ABL/ABL quantification may help in making early therapeutic decisions in CML specially after molecular relapse. However standardization is an important step to compare treatment in different groups. It is also helpful in the identification of various molecular subtypes on the basis of break point cluster regions involved in the translocation of the affected chromosome. A large study design and large sample size is required to arrive at a definite conclusion.

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