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MONITORING MINIMAL RESIDUAL DISEASE IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS: A PROSPECTIVE FOLLOW-UP STUDY

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Abstract—*Monitoring of minimal residual disease (MRD) is an integral part for evaluation in chronic myeloid leukemia (CML). The estimation of leukemic burden has a great impact on the estimation and decision making of treatment strategies, because of treatment failure in more than 25% of CML patients. Cytogenetic and molecular analyses have resulted in significant improved diagnosis and clinical decision-making. The response of CML patients to imatinib and other tyrosine kinase inhibitors is also taken into account during MRD monitoring and clinical follow-up.*

In the present study, 78 newly diagnosed CML patients positive for t (9;22)(q34;q11) translocation (Philadelphia chromosome) and initiated with imatinib therapy were monitored for a period of 24 months using conventional G-banding, FISH and qRTPCR. A majority of patients responded to the drug and achieved major molecular response (MMoR) by the end of the study. Four patients who did not respond to imatinib by the end of the study were recommended for autologous hematopoietic stem cell transplantation (HSCT). In summary, MRD using molecular approaches may identify patients that fail to respond to imatinib therapy, and may be suitable for HSCT.

Keywords*: Chronic myeloid leukemia, MRD, Imatinib, fluorescence in situ hybridization.*

1. INTRODUCTION

Chronic myeloid leukaemia (CML) is primarily due to excessive proliferation and failure of apoptosis of myeloid cells [1]. In India, it is one of the most common leukemia with an incidence of 0.8-2.2 per 100,000 population [2]. The incidence of CML varies according to ethnicity; it is 1.75 for Americans, 1.2 for Australians, and 0.64 for Europeans [3].

More than 90% of CML patients harbor the balanced translocation of chromosome 9 and 22, t(9; 22)(q34;q11), that results in the constitutive translation of a novel chimeric protein, p210BCR-ABL, that is responsible for dysregulation of cell proliferation and differentiation. Imatinib, a tyrosine kinase inhibitor (TKI) that targets BCR-ABL has become the first line treatment of choice in newly diagnosed CML in chronic phase, accelerated phase disease and even in blast crisis [1]. TKIs were approved by the Food and Drug Administration in year 2001 as main therapeutic agents for CML, improved the 8-year survival rates of CML patients to ~85% [4]. Imatinib has been available to Indian patients since 2002 and has changed the scenario of health care status especially in patients with CML [3]. However, a significant proportion of patients experience drug resistance and therefore, treatment failure. Studies across India have reported mutations in *bcr-abl* as the main cause of drug resistance [3]. A study reported nine common mutations, in 41% samples that accounted for 85% of the total mutations seen in the kinase domain of the *bcr-abl*, a frequency very different from that reported in the Western population [5]. With the emergence of better and more efficient therapeutic agents e.g., second generation TKIs, it is now possible for successful treatment of CML patients that develop resistance to imatinib.

The term "minimal residual disease" (MRD) is referred as the presence of residual neoplastic cells below the threshold of conventional morphological detection. Monitoring of MRD plays a critical role in the early detection of relapse of CML, which helps in timely therapeutic intervention and improved clinical outcome. Optimal use of therapeutic regimen for TKIs requires integration of molecular diagnosis and clinical investigations with regular follow-up. Therefore, the response to TKI is measured at several levels, i.e., conventional cytogenetic response (using Karyotyping and FISH), and Molecular Response using reverse-transcriptase-polymerase-chain-reaction (qRT-PCR). For MRD assessment of CML patients, regular monitoring of *bcl-abl* transcript levels has become the standard of care protocol for CML patients along with cytogenetic response for *bcl-abl* fusion, a major prognostic factor for disease progression and overall survival in CML.

For the present study, 78 untreated CML patients and diagnosed with Philadelphia chromosome were enrolled. They were initiated with imatinib therapy and for followed up for 2 years using both cytogenetic and molecular methods. using conventional G-banding, FISH and qRTPCR every 6 months. While 37% of the CML patients achieved major molecular response (MMoR) within one year, 4 patients (~5%) did not respond even after 24 months and were recommended for autologous hematopoietic stem cell transplantation (HSCT).

2. MATERIALS AND METHODS

The adult patients diagnosed with CML at Indraprastha Apollo Hospital, New Delhi, during the period from 2010 to 2015, diagnosed with CML on the basis of clinical and hematological criteria and with confirmed presence of *bcr-abl* were enrolled for the study. The written informed consent form was taken from all patients and the study was approved by Institutional Ethical Committee of Indraprastha Apollo Hospitals, Sarita Vihar, New Delhi. At diagnosis, all patients underwent complete physical examination, complete hemogram, and hepatic and renal function tests. All the patients were started on Imatinib (oral dose of 400 mg OD). After starting the treatment blood counts were performed weekly until patients achieved complete hematological response (CHR) and then monthly. The patients were also monitored for any potential side-effects along with molecular response to treatment with Imatinib. The dose of Imatinib was increased if patients showed evidence of disease progression. All patients underwent quantitative assessment of BCR-ABL ratio by reverse-transcription polymerase chain reaction (RT-PCR) method at baseline and every 6 monthly. Cytogenetic studies and FISH analysis of bone marrow cells were performed routinely at follow-up. Standard criteria for CHR, which included normalization of blood parameters and disappearance of clinical symptoms and signs including splenomegaly, major molecular response (MMoR) was defined as BCR-ABL/ABL ratio of less than 0.05% or 3 - log reduction from the baseline transcript values, whereas complete cytogenetic response and molecular response was defined as absence of t(9;22) and undetectable levels of BCR-ABL transcripts respectively.

Of the iniltally enrolled CML 78 patients enrolled for the study, 69 are still active and are on regular follow-up.

2.1 Karyotyping

Heparinized bone marrow and/or peripheral blood samples were collected in syringes or test tubes and sent to the laboratory at room temperature. Two different cultures (for 24-hour culture, and 48-hour culture) were prepared from these bone marrow samples. Culture media contained RPMI 1640 medium, 20% FCS (fetal calf serum), L-glutamine and penicillin/streptomycin (50 IU/ml and 50 µg/ml, respectively). Metaphases were harvested by adding colcemid (10µg/ml) solution followed by hypotonic KCl (0.075 M) treatment and fixation using standard 3:1 methanol: glacial acetic acid fixator. We used the conventional Giemsa banding (GTG banding) technique [6]. Five to ten slides were screened in each case and 10 – 20 metaphases were analyzed for each sample. At least three cells were karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN 1995) [7]. Analysis was carried out using a BX51 Olympus microscope and images captured with an automated image analysis system (Cytovision, Applied Imaging). A representative image is represented in Figure 1A.

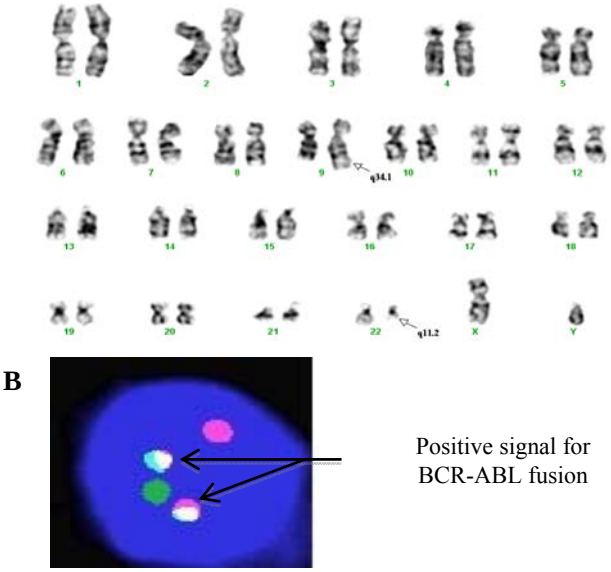

Figure 1: Representative images of samples positive for BCR-ABL fusion as determined by (A) karyotyping, and (B) FISH.

2.2 FISH analysis

FISH was carried out by standard protocol and hybridization procedure was modified according to the probe manufacturer (Vysis-Abbott Molecular Abbott Park, Illinois, U.S.A). Following hybridization excess and unbound probe was removed by the series of washes as recommended in the protocol. Finally, chromosome and nuclei were counterstained with DNA specific stain DAPI (4,6 diamidino-2-phenylindole) that fluoresces blue. A representative FISH result positive for *bcr-abl* fusion is represented in Figure 1B.

2.3 Real Time PCR studies

The commercial GeneXpert BCR-ABL monitor assay kit (Cepheid) was used according to the manufacturer's protocol. Briefly, the automated procedure is as follows: (i) purification of RNA using nucleic acid purification beads, (ii) reverse transcription of RNA, and (iii) quantitative real-time nested polymerase chain reaction of complementary DNA. For the assay, 200µl of blood or bone marrow aspirate was lysed immediately and run on the GeneXpert instrument. Wild-type

ABL transcripts served as an internal control. For positive specimens, the % *BCR-ABL* to *ABL* transcript was calculated by the following equation: % *BCR-ABL*/*ABL* = $E\Delta\ Ct^{\Delta Ct}$; where *Ct* is the cycle threshold, $E\Delta Ct$ is the efficiency of the *BCR-ABL* to *ABL* RQ-PCR reaction for a given lot of reagent, and $\Delta Ct = ABL\ Ct - BCR\text{-}ABL\ Ct$. For specimens negative for the *BCR-ABL* transcript, the detection limit was calculated as follows: % *BCR-ABL*/*ABL* detection limit = $E\Delta Ct^{ABL\ Ct}$. A representative data for successful therapy is shown in Figure 2.

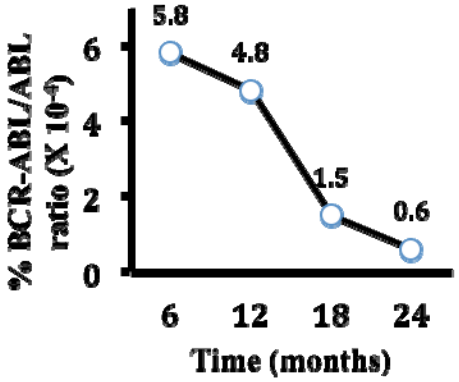

Figure 2: Assessment of BCR-ABL transcript levels for MRD in successful therapy

3. RESULTS

The demographic and clinical features are presented in Table 1. The age varied between 18 and 66 years, median age was 39 years. Nearly 51% of patients presented with splenomegaly, 31% had hepatomegaly whereas only 3% had peripheral lymphadenopathy.

Table 1: Demographic and Clinical features of CML Patients

Clinical Parameter	Number (Frequency)
Male Female	58(74.35%) 20 (25.64%)
Mean Age	39 yrs
Spleenomegaly	40 (51%)
Hepatomegaly	24 (31%)
Lymphoadenopathy	2(3%)
Pallor	16 (20%)
Mean Hb (gm)	11
Mean WBC	40,000/mm^2
Mean Platelet	500000

Complete haematological response was seen in 97% of patients. The median time to attainment of CHR was 4.3 Weeks (Range from 2 weeks to 2½ months.). Complete cytogenetic response was seen in 97% of patients with a median time to attainment of CCR was 15 months (range 12-24 months).

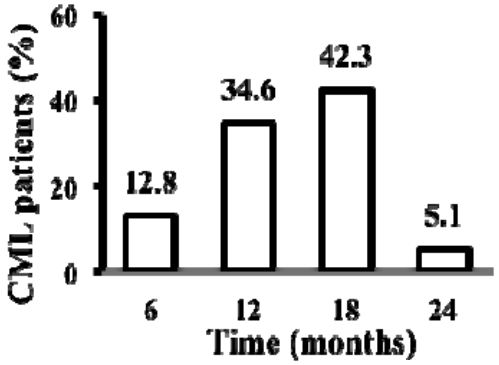

Figure 3: Time taken for complete MMoR

Monitoring of BCR-ABL transcript levels revealed that 12.8% of patients had achieved MMoR by 6 months and another 34.6% patients achieved MMoR by 12 months. By 18 months, 42.3% more patients had achieved MmoR, and by 24 months, all but 4 patients had achieved MmoR (Table 2 and Figure 3). Patients not responding to imatinib were given autologous HSCT.

Table 2: Molecular Response of CML patients

Time to achieve MMoR (months)	Number of patients (Frequency)
6	10 (12.8%)
12	27 (34.6%)
18	33 (42.3%)
24	4 (5.1%)

At the end of the study, 4 CML patients were identified who failed to respond to imatinib therapy and were recommended for autologous HSCT. A comparison of the three methods for MRD (Table 3 and Figure 4) revealed that cytogenetic analysis by either karyotyping or FISH was able to identify only 2 patients each that were negative for BCR-ABL. Interestingly, the two patients determined to be negative for BCR-ABL by karyotyping were found to be positive by FISH, and *vice-versa*. Further, qRTPCR-based assay for BCR-ABL levels identified all 4 patients as non-responders for imatinib therapy.

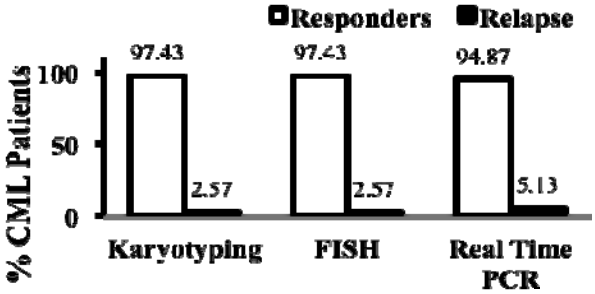

Figure 4: Response to imatinib therapy at the end of 24 months using 3 different methods of assessmentA

Table 3: Comparison of Karyotyping, FISH and Real Time PCR for BCR-ABL in CML patients

Technique	Number (Frequency) after Imatinib Treatment	
	Negative (Responders)	Positive (Relapse)
Cytogenetics	76 (97.43%)	2 (2.57%)
FISH	76 (97.43%)	2 (2.57%)
Real Time PCR	74 (94.87%)	4 (5.12%)

4. DISCUSSION

The introduction of TKIs such as imatinib has revolutionized the treatment of CML with significant improvement in the survival rate for patients. The progression of CML includes three distinct phases: chronic phase to accelerated phase to blast crisis. If left untreated CML patients progress to the blast crisis state and succumb to the disease within a year [1]. When put on imatinib therapy, CML patients remain in the chronic phase for a very long duration of time followed by the accelerated phase; however, they rarely progress to the blast crisis stage.

In the present study, patients were included with median age of 41yrs (range 18-66yrs) with (male female ratio of 2.9:1) which is in concordance with previous studies published from this subcontinent. Similar results of male preponderance has been reported earlier [8-12].

After initiating the treatment, the first step is to check the hematologic parameters followed by cytogenetic and molecular criteria. In line with previous reports [8-10], we noted hematological remission in 95% of CML patients after 24 months on imatinib. From all the three methods used in the study, molecular response assessment by quantitative RT-PCR was observed to be most specific and sensitive for CML patients. RT-PCR can be performed easily using either bone marrow or peripheral blood samples. Molecular monitoring using RT-PCR is a very sensitive technique and can measure disease burden even in patients who have achieved complete cytogenetic remission; it is also helpful for identifying patients who are at higher risk of resistance or relapse [1]. Studies have shown that BCR-ABL/ABL ratio is an accurate surrogate marker for the contemporary marrow cytogenetic response in patients treated with imatinib; early trends in BCR-ABL/ABL ratio are helpful in predicting cytogenetic response after 6 months of therapy [13].

In our study, 47% of CML patients achieved MMoR s by 12 months; by 18 months, another 36% had achieved MMoR. Previous studies have found that 37.5% patients achieved CMoR in first 6 months and remaining patients who did not achieve CMoR at 6 months failed to achieve even at 1 year also, supporting the fact that molecular response is an ongoing process, and there exists a proportion of patients responding slowly but still achieving molecular responses even later than 48 months period. Another study from north India showed MMoR of 32% [1, 14].

The molecular response markers are useful for determining disease prognosis, but are also able to predict response to specific drugs. For example, CML patients who achieve early MMoR also have durable cytogenetic responses along with better progression-free survival. The assessment of molecular response can be preformed using peripheral blood and avoid the need for bone marrow biopsy which is a painful, invasive technique which almost all of patients are reluctant to undergo periodically [1].

However, cytogenetic testing may be considered for special situations like increasing BCR-ABL transcripts, which may indicate loss of response and may help in detecting clonal evolution. In summary, monitoring molecular responses in CML patients is a good parameter especially in developing countries like India. Furthermore, it can be used for timely decisions regarding the investigative and management protocols besides predicting the therapeutic outcome.

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