Diagnosis and disease management in CML patients using conventional and molecular cytogenetics

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Abstract
Chronic Myeloid Leukemia (CML) is a hematopoietic malignancy characterized by the presence of Philadelphia (Ph1) chromosome that results from balanced reciprocal translocation between chromosomes 9 and 22 leading in the formation of bcr/abl fusion gene. The present study was conducted to evaluate cytogenetic and molecular abnormalities in CML patients at presentation and during the course of therapy. Cytogenetic analysis was carried out in bone marrow samples of 165 suspected patients of CML using standard protocols. Sequential cytogenetic analysis was also done in 55 CML patients (50 on IFN-α 2b therapy and 5 on Hydroxyurea) up to variable period of 3 years. Fluorescence In Situ Hybridization (FISH) using specific probes for bcr and abl genes was carried out in cases where conventional cytogenetics was not informative, in cases that were Ph-negative at presentation and in cases with complete or major cytogenetic response (<34% Ph+cells). Of the 165 CML patients, 157 patients (95%) were Ph-positive while 8 patients (5%) were Ph-negative. Cytogenetic abnormalities other than the standard Ph1 translocation were observed in 2 Ph+ patients and 2 Ph-negative patients. Sequential cytogenetic analysis revealed varied degrees of cytogenetic response in 35 patients on IFN-α 2b therapy. Complete cytogenetic response was observed in 8 patients (16%) on IFN-α 2b therapy. However, FISH analysis could detect bcr/abl fusion gene in some of these cases. This finding highlights the sensitivity of this technique in detecting residual disease even in patients with complete cytogenetic remission. The other patients in complete cytogenetic remission did not have evidence of bcr/abl fusion. FISH analysis also revealed bcr/abl fusion signals in Ph-negative cases (with no cytogenetic abnormality) indicating a masked translocation or a sub-microscopic rearrangement.

Thus, the results of the present study show that analysis of cancer patients on therapy at the molecular level has tremendous importance for management of CML patients. Further, with the use of highly sensitive FISH technique, results can be obtained within 24 hours thereby, aiding rapid diagnosis and management of these patients. This efficient and highly sensitive molecular cytogenetic technique can also be done on interphase cells and poorly spread metaphases thereby, overcoming the difficulty of conventional chromosomal analysis especially in patients on therapy.

Keywords: CML, Diagnosis, Cytogenetics, FISH

INTRODUCTION
Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder resulting from a neoplastic transformation of primitive hematopoietic stem cells of bone marrow. The cytogenetic hallmark of the disease is the presence of Philadelphia (Ph1) chromosome that results from a balanced reciprocal translocation between long arms of chromosomes 9 and 22, t (9; 22) (q34; q11) (Nowell and Hungerford 1960; Rowley 1973). At the molecular level, this translocation leads to the repositioning of the Abelson (abl) protooncogene from its normal position on 9q34 to the breakpoint cluster region (BCR) on 22q11 resulting in the formation of a novel bcr/abl fusion gene that expresses an abnormal fusion protein with elevated tyrosine kinase
activity (Groffen et al. 1984; Shtivelman et al. 1985; Silver et al. 1999) About 90-95% cases have the Ph1 chromosome while 5-10% are Ph-negative and may have masked translocation or complex translocation (Nowell and Hungerford 1960; Shtivelman et al. 1985).

CML is basically a biphasic disease that progresses from a benign chronic phase to a terminal blast crisis that may be preceded by an ill-defined accelerated phase. The progression of the disease from blast crisis to accelerated phase or blast crisis is accompanied by the appearance of additional cytogenetic abnormalities (+8, -7, i (17q), -Y etc.) in 60-70% cases (Nowell and Hungerford 1960; Groffen et al. 1984; Shtivelman et al. 1985). About 10% cases in chronic phase have also been reported to have these additional cytogenetic abnormalities (Shtivelman et al. 1985; Kantarjian et al. 1995).

The present-day management of CML that aims at eradicating the malignant clone harboring the genetic defect rather than just controlling abnormal blood counts has brought interferon-based regimens to the limelight (Kantarjian et al. 1995; Chomel et al. 2000). Various clinical trials have shown that Interferon-α (IFN-α) alone or in combination with other myelo-suppressive drugs can induce complete hematological remission in about 70% to 80% CML patients and complete cytogenetic response in up to 26% of the patients treated at diagnosis (Kantarjian et al. 1995; Chomel et al. 2000; Hochhaus et al. 2000; Acar et al. 1997; Hochhaus et al. 2000). Complete responders survive significantly longer than those without cytogenetic response but some patients ultimately relapse with the reappearance of the Ph-positive clone. However in most of these cases, persistence of \textit{bcr/abl} fusion gene has been demonstrated by highly sensitive molecular methods (FISH, RT-PCR etc.) (Acar et al. 1997; Hochhaus et al. 2000). The frequency of \textit{bcr/abl} positive cells in such cases differed in different studies. The evaluation of the tumor burden reduction that has become the primary concern of the clinicians is known as minimal residual disease (MRD), which is also an important prognostic factor and detects early progression of disease (Hochhaus et al. 2000).

The present study was conducted to evaluate the cytogenetic and molecular abnormalities in CML patients at diagnosis and during the course of therapy using conventional cytogenetics and Fluorescence In Situ Hybridization (FISH).

**MATERIALS AND METHODS**

Two hundred and thirty-seven bone marrow samples from 165 CML patients (114 males and 51 females) were analyzed by conventional and molecular cytogenetics. The median age of diagnosis was 38 years (range: 2 years to 75 years). Sequential cytogenetic analysis was also carried out up to variable period of 3 years in 55 of these patients (50 on IFN-α 2b therapy and 5 on Hydroxyurea). FISH analysis was done on interphase nuclei and metaphases in cases with complete or major cytogenetic response (< 34% Ph+ cells), in cases that were Ph-negative at presentation and in cases where no analyzable metaphases were obtained by conventional cytogenetics.

**Cytogenetic analysis:** Cytogenetic analysis was performed on bone marrow aspirates of all the 165 suspected cases of CML at presentation. Of these 50 patients on IFN-α therapy and 5 on Hydroxyurea (HU) were followed up to a variable period of 3 years (ranging from 8 to 36 months). The response criteria used for assessing the cytogenetic response in these patients on IFN-α therapy were the standard criteria defined in earlier studies (Table 1).

Chromosomal spreads were obtained from unstimulated 24-hours culture of bone marrow aspirates using standard protocols (Roulston, D. and Beau, M.M.I. 1997).

Karyotyping was done on GTG-banded metaphases by both automated and manual methods following ISCN nomenclature (ISCN 1995). Karyotyping was also done on C-banded metaphases using standard protocol in 2 cases (Roulston D. and Beau M.M.I. 1997).

**Fluorescence in situ Hybridization (FISH) analysis:** FISH analysis was performed on interphase nuclei and metaphases obtained from 40 bone marrow aspirates using Vysis LSI \textit{bcr/abl} extra signal (ES) translocation probe. This commercially available probe produces 2 green and 2 orange signals in a normal cell representing native \textit{brc} gene (chromo-
some 22) and native abl gene (chromosome 9) respectively. In a cell possessing the bcr/abl translocation, a yellow signal representing bcr/abl fusion gene and an additional orange signal on derivative chromosome 9 are present apart from one green (bcr) and one orange (abl) signals. The use of this ES probe reduces the interpretation problems resulting from random juxtapositioning of differently labeled genes producing co-localized signals (Froncillo et al. 1996; Werner et al. 1997). The probe was standardized on peripheral blood samples obtained from normal healthy individuals and bone marrow samples obtained from CML patients and patients with hematologic disorders other than CML before using it for the study.

The slides prepared from fixed cell suspension obtained during cytogenetic analysis were denatured in 70% formamide/2X SSC at 73°C for 2-5 minutes. The slides were then dehydrated ethanol (70%, 85% and 100%) series. After the slide was dried, 10µl of denatured probe was applied to the marked area of the slide with highest cell density. Hybridization was carried out overnight at 37°C in a humidified chamber. Post-Hybridization washes were given in 0.3% NP-40/0.4X SSC (73°C; 2 minutes) followed by 0.1% NP-40/0.4X SSC (room temperature; 1 minute). The slide was then counterstained with DAPI and viewed under a Zeiss Axioskop fluorescence microscope. Images were captured using a CCD camera attached to the microscope and analyzed using CytoVision software (Applied Imaging, UK). A minimum of 200 interphase nuclei and 20 metaphases wherever possible was scored for each case. Clumped nuclei and nuclei having signals with low fluorescence intensity were excluded (Jobanputra et al. 1998).

**RESULTS**

Of the 165 patients analyzed by conventional cytogenetics, 157 patients (95%) were Ph-positive while 8 patients (5%) were Ph-negative. Among the 157 Ph+ patients, 139 were 90-100% Ph+ while 17 patients were 50-80% Ph+. Further, 1 patient had the standard translocation, t (9; 22) (q34; q11) in only 18% metaphases (Figure 1). Two Ph-positive patients had additional cytogenetic abnormalities: one had a complex translocation, t (1; 9; 15; 22) (q24; q34; q15; q11) in 10% metaphases and del (Y) in another 10% metaphases while the other patient had duplication of Ph1 chromosome in 20% metaphases.

Of the 8 Ph-negative patients, conventional cytogenetics revealed cytogenetic anomalies in 2 cases while FISH analysis revealed the presence of bcr/abl fusion gene in another 2 cases (Figure 2). Of the 3 Ph-negative patients, one patient had der (17) and loss of Y chromosome in 10% and 15% metaphases respectively.

Sequential cytogenetic analysis in the 50 patients on IFN-α 2b therapy revealed varied degrees of cytogenetic response: complete cytogenetic response (CCR) was achieved by 8 patients (16%), partial
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Figure 1: G-banded karyotype showing t(9;22) (q34;q11). Arrows indicate der (9) and der (22).

Figure 2: Identification of bcr/abl rearrangement in interphase nucleus and metaphase chromosomes from bone marrow sample of CML patient using FISH analysis. (a) bcr/abl positive nucleus showing bcr (green), abl (red), der (9) (extra red signal or es) and bcr/abl fusion gene (red/green) (b) Metaphase showing bcr/abl fusion signals (yellow).
DISCUSSION

Cytogenetic analysis is the ‘gold standard’ for genome-wide screening of cytogenetic abnormalities. However, it is often difficult to obtain well-spread metaphases with good chromosome morphology from bone marrow samples of CML patients especially on IFN-α therapy due to which at times, analysis is difficult using conventional cytogenetics (Hochhaus et al. 2000; Hochhaus et al. 2000). Molecular cytogenetics or Fluorescence In Situ Hybridization (FISH) that can be done on interphase nuclei, poorly spread metaphases and well-spread metaphases plays an important role in such conditions for diagnosis and evaluation of minimal residual disease (MRD)s (Kantarjian et al. 1995; Hochhaus et al. 2000; Werner et al. 1997; Jobanputra et al. 1998; Cortes et al. 1999). In the present study, bone marrow samples from 6 patients (3 at diagnosis and 3 during IFN-α 2b therapy) that could not be analyzed using conventional cytogenetics were analyzed for the gene rearrangement using FISH on interphase cells and poorly spread metaphases.

Using FISH technique, bcr/abl gene rearrangement was detected in 2 cases where no cytogenetic evidence of the standard translocation (i.e. no Philadelphia chromosome) was present. This finding highlights the high sensitivity of the FISH technique indicating that FISH analysis should be used in all CML cases with no evidence of Philadelphia chromosome. Further, this highly sensitive FISH technique can detect an abnormal clone of even small size (1-2 cells in 100 cells), which could be missed by conventional cytogenetics (Hochhaus et al. 2000; Reiter et al. 1997; Takahashi et al. 1996; Cortes et al. 1995). In the present study, 4 patients who achieved complete cytogenetic response (0% Ph+ cells) as a result of IFN-α 2b therapy revealed bcr/abl fusion signals using FISH on metaphases and interphase nuclei. This emphasizes on the importance of using FISH analysis for evaluation of minimal residual disease in CML patients on IFN-α therapy.

How the molecular event occurred without the cytogenetic findings is not yet known. Some studies suggested a double translocation mechanism where the first event is the standard Ph translocation followed by a second translocation between der (9) and der (22) which masks the first chromosomal exchange thus resulting in a normal karyotype (Cortes et al. 1999; Takahashi et al. 1996; Tanaka et al. 2000). Others suggested that a small segment of chromosome 9q34 might be inserted into chromosome 22 in the middle of the bcr gene (Jobanputra et al. 1998; Reiter et al. 1997; Cortes et al. 1995). Some studies also suggest that these bcr/abl positive cells in the IFN-α responders may represent residual lymphocytes or non-proliferating neoplastic cells (Chomel et al. 2000; Froncillo et al. 1996; Werner et al. 1997). Regardless of the mechanism, the present findings support the relevance of the chimeric bcr/abl gene in the pathogenesis of CML.

It is possible that these residual rearranged cells i.e. bcr/abl positive cells kept in a “dormant state” probably mediated by immune mechanisms of IFN-α 2b re-enter the cell cycle leading to the expansion of the malignant clone (Froncillo et al. 1996; Takahashi et al. 1996). Thus a potential risk of relapse exists for CML patients as long as their cells have the underlying molecular defect i.e. bcr/abl gene rearrangement (Chomel et al. 2000). Such risks of relapse reinforce the need for sequential cytogenetic and molecular analysis in CML patients on therapy.

The remaining 4 CML patients out of the 8 patients...
on IFN-α 2b therapy who achieved complete cytogenetic response did not show any evidence of bcr/abl positive cells using FISH analysis. This finding implies a complete response in these patients both at the cytogenetic and molecular levels and raises the question whether IFN-α 2b therapy actually cures the disease. These findings, however, do not exclude the possibility of low level mosaicism and suggest a need for more follow ups to see whether such a response is permanent or not.

Though targeted FISH is a rapid and highly sensitive technique, it cannot be used for genome-wide screening like conventional cytogenetics (Werner et al. 1997; Jobanputra et al. 1998). This was reflected in the cases that were Ph-negative and also lacked bcr/abl rearrangement at presentation but had other cytogenetic abnormalities (der (17 and −Y).

Thus, FISH analysis should be used as a supplement to conventional cytogenetics for detecting the rearrangement at the molecular level especially in Ph-negative CML cases and for evaluation of minimal residual disease in patients achieving cytogenetic response as a result of therapy. Further, the use of FISH on interphase and metaphase cells can provide results within 24 hours that can facilitate rapid diagnosis and management of these patients.

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