

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 (Ph^1) 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 Ph^1 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 (Ph^1) 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

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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 *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 *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 *bcr/abl* extra signal (ES) translocation probe. This commercially available probe produces 2 green and 2 orange signals in a normal cell representing native *bcr* gene (chromo-

Table 1: Standard Criteria for response to IFN- α in CML.

Response	Type of response	Criteria
Hematological response	Complete	Normalization of WBC counts to <9000/ μ l, normal differentials, normalization of platelet count to <450000 μ l, disappearance of signs and symptoms of disease
	Partial	Normalization of WBC with persistent splenomegaly or immature peripheral cells or thrombocytosis <50% of pretreatment
Cytogenetic response	Complete*	0% Ph+ metaphases
	Partial*	5% to 34% Ph+ metaphases
	Minor	35% to 90% Ph+ metaphases
	No response	Ph+ metaphases > 90%
Molecular response	Complete**	<i>bcr/abl</i> negative cells
	Residual disease	clone of <i>bcr/abl</i> positive cells

References: Silver *et al.* 1999; Kantarjian *et al.* 1995; Hochhaus *et al.* 2000; Cortes *et al.* 1999). * Major cytogenetic response = complete or partial cytogenetic response (< 35% Ph+ metaphases). ** Below the cut-off value (i.e. 10 in the present study).

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, 10 μ l 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 Axiophot 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

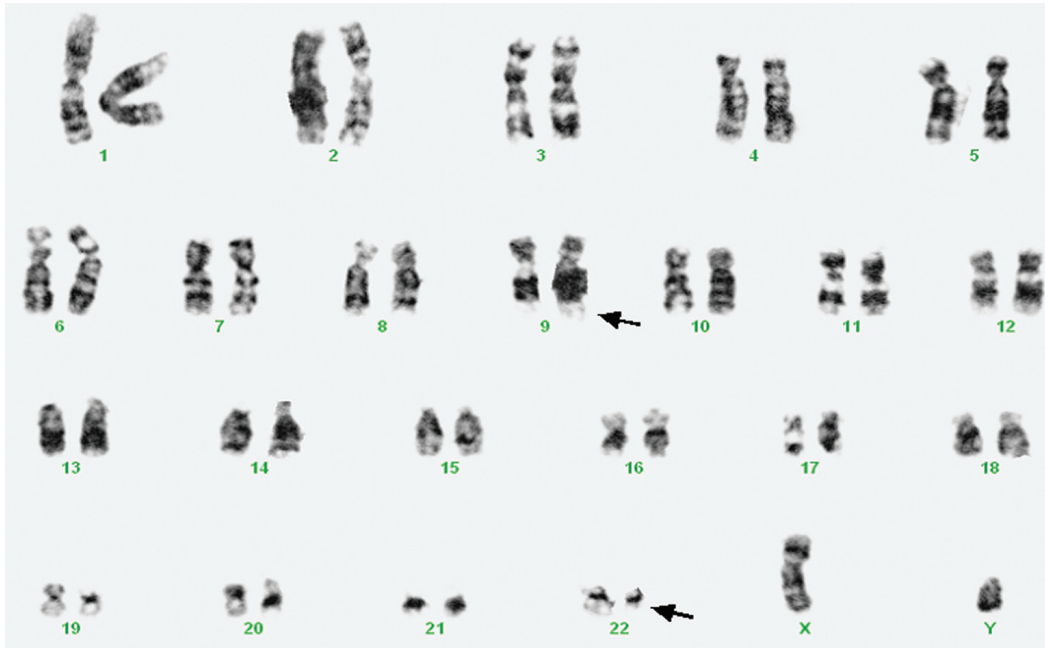


Figure 1: G-banded karyotype showing t(9;22) (q34;q11). Arrows indicate der (9) and der (22).

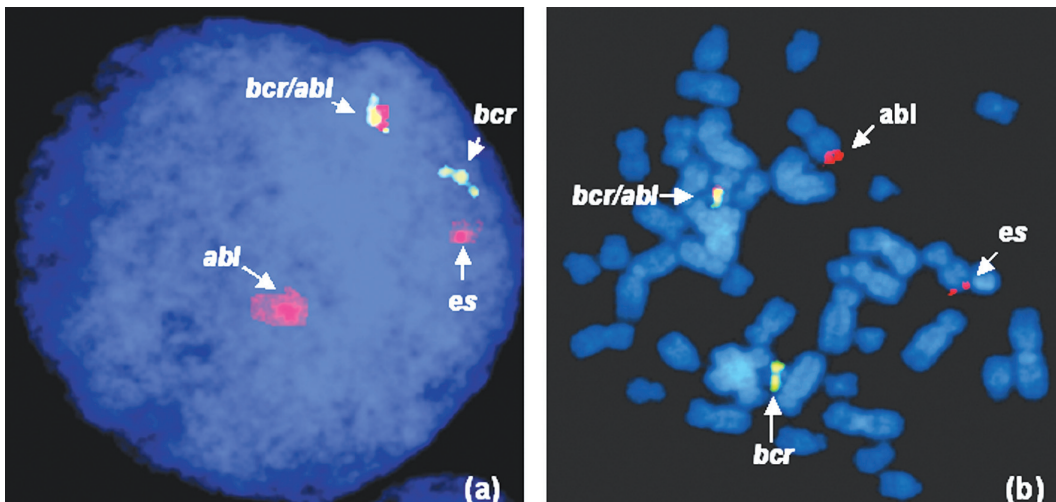


Figure 2: Identification of *bcr/abl* rearrangement in interphase nucleus and metaphase chromosomes from bone marrow sample of CML patient using FISH analysis. (a) *bcr/abl* positive nucleus showing *bcr* (green), *abl* (red), der (9) (extra red signal or es) and *bcr/abl* fusion gene (red/green) (b)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

cytogenetic response was achieved by 3 patients (6%), minor response was seen in 24 patients (48%) while 15 patients (30%) did not show any response. No cytogenetic response was observed in any patient on Hydroxyurea. The time of achieving the response varied in different cases. Of the 8 patients on IFN- α 2b therapy with complete cytogenetic response (0% Ph⁺ cells), FISH analysis revealed *bcr/abl* rearrangement in 80-100% cells in 4 patients. The remaining 4 out of the 8 patients in CCR did not have the *bcr/abl* rearrangement at the molecular level.

Due to poor quality of metaphase spreads, cytogenetic analysis was not informative in 6 bone marrow samples from 3 patients at diagnosis and 3 patients during IFN- α 2b therapy. These samples were then analyzed using FISH on interphase nuclei. FISH analysis revealed *bcr/abl* fusion signals in 5 samples (3 at diagnosis and 2 on IFN- α therapy) indicating presence of the malignant clone. However, one patient on IFN- α therapy did not reveal any *bcr/abl* fusion signals implying a complete cytogenetic and molecular response.

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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