

# Monitoring the Course of Chronic Myelogenous Leukemia by Fluorescence *In Situ* Hybridization

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## 1. Introduction

In situ hybridization is based on the base pairing of the DNA probe to complementary sequences in cells of the sample. Fluorescence detection has been the most popular technique for a variety of reasons including its high sensitivity, signal resolution, and, most of all, the potential to simultaneously detect multiple target regions [1,2]. The different types of DNA probes useful for clinical applications can be categorized according to the complexity of the respective target sequences. Most genomic DNA fragments cloned in plasmid, phage, cosmid, P1, or yeast artificial chromosome (YAC) vectors, detect unique loci in the genome. To reach efficiencies needed for diagnostic applications, probes comprising at least 20-50 kB (i.e., cosmid size probes) are required. Larger chromosomal regions and even whole chromosomes can be "painted" by using probe pools derived from for examples, flow-sorted or microdissected chromosomes, or from somatic hybrid cell lines containing only one human chromosome or segments thereof [3-5]. In this review, recent advances in the analysis of CML made by fluorescence in situ hybridization (FISH) studies and future prospects will be discussed.

## 2. Value of Metaphase-Fluorescence in Situ Hybridization (M-FISH) to Detect Variant Ph Translocation

Variations of the typical Ph (VPh) translocation occur in 3% of CML cases [6,7] ; VPh-CML is often difficult to detect by standard cytogenetic procedures, and the incidence of VPh may be higher than suspected. Chromosome-specific probes using dual Alu primers to direct

polymerase chain reaction (PCR) amplification of human DNA from hybrid cells containing regions of the human genome (i.e., chromosomes 1, 9, and 22) in rodents were generated. Because FISH probes made in this manner do not recognize centromeric sequences [8], such probes could be used to delineate complex VPh chromosomes rapidly and unambiguously. This is especially true in translocations involving multiple chromosomes (i.e., variant Ph-positive CML involving chromosome 1, 9, and 22), for which FISH with inter-Alu PCR probes should allow accurate and fast analysis of chromosome abnormalities [9]. It is important that the method identifies these events in the less than ideal metaphases that often occur in clinical samples and in which the abnormalities are difficult to resolve by standard cytogenetic procedures.

## 3. Analysis of Interphase Cells for the Philadelphia Translocation by FISH

Identification of the Ph chromosome in interphase nuclei had been first attempted using combination of cosmid probes [10]. Digitized images were collected with a cooled charged coupled device (CCD) camera and displayed after computer enhancement. A combination of the two colors indicated the presence of the Ph chromosome. The small size of the region detected by these probes raises questions as to their reliability for large-scale clinical use. Inter-Alu-PCR product from the YAC showed that 64% of the interphase nuclei from the bone marrow (BM) of a CML patient contained more than two signals, whereas 96% of normal diploid cells had two signals or less, as expected [11]. Because these signals appeared as dots in the interphase nuclei, there was a possibility of false negatives (mis-

sing some true targets because of incomplete hybridization) or false positives (counting artificial spots). However, in a sufficiently large sample size, it was suggested that the presence of cancer cells in a population could be detected.

We examined the usefulness of a larger size probe derived by inter-All-polymerase chain reaction of DNA from an interspecific somatic cell hybrid containing approximately 5 MB of human DNA covering the ABL gene region on human chromosome 9q34 to give a clear, yet resolvable signal for detection of three domains of hybridization in CML interphases and two domains in normal cells. We also evaluated the effectiveness of combining that probe with a BCR probe in two-color procedures for high-efficiency quantitation of the frequency of Ph-positive cells. Such quantitation enables the detection of Ph-positive cells in heterogeneous cell populations with obvious application to research and management of the disease [12].

An experiment was performed with a subset of samples from the previous series—five normals and five CML patients. All interphase cells (not just polys) were counted (three domains: 79.2%; two domains: 20.6%). However, interphase FISH with E6B does have its limitations. At present, it appears that the signal generated by the large probe, although very efficient for resolving the domains in polys, is often diffuse in other cell types that may include more immature cycling cells. In addition, about 5% of the cells from normals and 9% of the cells from CML patients were judged unscorable.

#### 4. Hypermetaphase Fluorescence in Situ Hybridization (HMF)

##### 4.1. Quantitative Monitoring of Ph-Positive Cells During Ifn- And Sti 571 Treatment

While extremely reliable when a good harvest of metaphases is obtained from a marrow culture, more than 20-25 metaphases are rarely recovered from patients on therapy. Consequently, in patients with CML receiving treatment, monitoring the effect of therapy by cytogenetic studies (CG) is limited with respect to detection minimal residual disease (MRD) and identifying significant changes in Ph-positive cell frequency. Analyzing greater numbers of metaphases per sample would provide higher resolution and greater efficiency in addressing these issues. Long-term exposure (24 hours) of bone marrow cultures to colcemid (0.1µg/mL) maximized the frequency of metaphase collection. Such preparations were subjected to FISH using a probe that overlapped the region of the translocation at chromosome 9q34. This detected the Ph translocation in the resultant large number of overly contracted chromosome spreads. The procedure was validated and verified by studying 70 double-blind marrow samples from patients in different stages of Ph-positive CML, and from patients with Ph-negative hematological malignancies (controls). This hypermetaphase FISH (HMF) method clearly identified Ph-positive metaphases and allowed the

analysis of 500 metaphases per sample in <1 hour after FISH [13]. In this analysis, 24% of patients were reclassified into a cytogenetic response category that was more appropriate than the one assigned by CG. It was also successful in providing response data in patients with insufficient metaphases for regular CG studies. Of significance was the ability of HMF to detect MRD in three of 19 patients in complete cytogenetic response by routine CG, thus providing insight into their true response status, which may influence treatment decisions (dose schedule, additional agents, need to continue therapy). Furthermore, in patients shown to be 0% Ph-positive by HMF on 500 cells, an argument could be made to reduce or stop IFN- therapy or STI 571 while continuing close monitoring by HMF. Studies on 500 cells have very narrow confidence intervals ( $\pm 2\%$ ) and minor changes (<4%) observed at periodic intervals (e.g., every 3-6 months) can be reliable measurements of the efficiency of a particular therapy and disease evolution.

The maximum frequency of cancer cells present at the indicated confidence levels (at 99%, 95%) were: 0.11, 0.007 when 0 cancer cells were seen in 400 metaphases; and 0.009, 0.006 when 0 cells were seen in 500 metaphases. Thus, in the standard HMF analysis, when 500 cells are studied and no cancer cell hypermetaphases are identified, there is a 99% confidence that there are <1% cancer cells in the sample. With respect to the assessment of minimal residual disease during therapy, a complete cytogenetic response by CG may in fact be only a partial cytogenetic response by HMF, or it may be a complete response of high quality (0 Ph-positive cells in 500 cells analyzed). This should help in decisions regarding continuation of IFN- $\alpha$  therapy and/or addition of other agents (e.g., low-dose cytosine-arabioside [ara-C]), dose schedule adjustment, or even discontinuation of therapy.

The major limitation of HMF is the inability of the procedure to detect cytogenetic clonal evolution, an indicator of worse prognosis [14], as it is focused on scoring the single cytogenetic event of interest (in this case, the Ph chromosome). This is also recent evidence that clonal evolution may not carry the same poor prognostic impact in such cases as when occurring with other features [15].

#### 5. Early Detection of Relapse by HMF after Allogeneic Bone Marrow Transplantation (BMT)

Using probes that identify Ph-positive cells, HMF was compared with standard CG in the follow-up evaluations of 51 patients with CML following allogeneic BMT (21.22). Among them, 12 (23.5%) were negative for Ph by standard CG but were positive by HMF. Among these 12 patients, six (50%) experienced CML relapse after median 103 days (range 63-185 days), and the 5-year actuarial survival was 28%. None of the other 35 patients who were Ph-negative by CG and HMF relapsed; their actuarial survival was 67% at 5 years. Early recognition of CML relapse post BMT is be-

coming increasingly important with the recent documentation that donor lymphocyte infusions (DLI) can reinduce complete remission via a graft-versus-leukemia effect in patients relapsing into chronic phase. This approach was most effective when applied early in the course of relapse. Kolb et al. [16] reported that among 84 patients, 14 (82%), 39 (78%), and one (12.5%) achieved complete remission after DLI for cytogenetic relapse, hematological relapse, and transformed phase relapse, respectively. Van Rhee et al. [17] reported a higher remission rate and a lower risk of marrow aplasia and other complications in patients transplanted early in cytogenetic relapse than in overt hematological relapse. Early intervention, when donor-derived hematopoietic cells are dominant, might prevent the complications associated with pancytopenia. We conclude that in patients with CML after allogeneic BMT, HMF should be considered as a quantitative technique to detect and monitor the results of therapy for CML.

## 6. Analysis of Ph-Negative Bcr-Positive CML by HMF

Approximately 5-10% of patients with a morphologic diagnosis of CML are Ph-negative [18]. They tend to be older (median age >65 years) and to have monocytosis, thrombocytopenia, a poor response to chemotherapy, and an overall shorter survival than Ph-positive CML patients. Thirty percent of them have rearrangement of BCR by Southern blot (Ph-negative BCR-positive) and are indistinguishable from patients with Ph-positive CML in relation to characteristics, response, and prognosis. We recently analyzed six such patients by HMF. Two of the six patients showed an insertion of 9q34 into chromosome 22q11 with a frequency of 74.2% and 92%. In both cases, the insertion was easily identified by HMF. In addition, HMF detected a low frequency of Ph-positive cells at diagnosis in two

patients (patients 2 and 5) and during hematological relapse in two other patients (patients 4 and 6). The pattern of UBCR was identical in the classical pattern in Ph-positive CML. The p210 results correlated well in two patients (patients 2 and 5) but were discordant in one (patient 2), in whom high p210 expression with of a low Ph-positive frequency might suggest the presence of a cryptic BCR-ABL junction. The HMF analysis was useful in identifying Ph-positive clones, in Ph-negative BCR-positive CML, and characterized the nature of the chromosome aberration, i.e., an insertion. It identified two distinct subsets of Ph-negative BCR-positive CML: one with an insertion of 9q34 into 22q11, the other with a low frequency of typical Ph chromosome translocations. The use of HMF with other complementary molecular studies (i.e., Southern and Western studies) in larger study groups may help further define the heterogeneity and prognosis in Ph-negative BCR-positive CML.

## 7. Blood Versus Bone Marrow Studies for Ph Monitoring by FISH

The technique for monitoring Ph frequency with HMF involves the invasive and painful procedure of bone marrow aspiration. One of our aims has been to develop a noninvasive, yet highly reliable, method to monitor the frequency of Ph-positive cells in CML patients undergoing therapy. By testing the use of interphase (I-FISH) on peripheral blood (PB), we chose to target polymorphonuclear leukocytes (polys), which have better resolution with I-FISH techniques. The I-FISH results (five normals and 26 CML) were compared with their respective bone marrow (BM) HMF results to verify the correlation between the frequency of Ph-positive cells obtained by the two methods.

The BCR/ABL translocation probe (Vysis) was used to detect BCR/ABL gene fusion, and this probe was

**Table 1.**

Summary of Standard Cytogenetic Studies, Southern and Western Blots and HMF in the Study Group.

Patients	Karyotype(CG)	BCR/ABL Rearrangement	P210BCR.ABL Expression	HMF(%)
1	46,XY[20]	positive	+	22/400(5.5%)—ch 9 29/500(5.8%)—ch 22
2	46,XY[23]	positive	++++	25/475(5.2%)—ch 9 22/500(4.4%)—ch 22
3	46,X,-Y[24]46,XY[1]	positive	Na	371/500(74.2%)—ch 9 0/500(0%)—ch 22
4	46,XY,del(19)(p13.2)[19]46,XY[1]	positive	Na	462/501(92%)—ch 9 0/800(0%)—ch 22
5	46,XY[20]	positive	+	17/400(4.2%)—ch 9 18/500(3.6%)—ch 22
6	46,XY[20]	positive	Na	2/800(0.3%)—ch 9 1/800(0.1%)—ch 22

highly effective in identifying Ph-positive cells in the majority of cells in PB polys. Our FISH probe containing only approximately 5 Mb of human DNA surrounding the ABL locus on human chromosome 9 probe was used to detect BCR/ABL gene fusions in BM metaphases. The percent Ph-positive by I-FISH was estimated using the method of Thall et al [20]. The correlation between the percent of Ph positivity by I-FISH on polys and percent of Ph positivity metaphases by BM HMF in 26 samples was excellent ( $r=0.983$ ,  $P<0.0001$ ). Peripheral blood I-FISH was not suitable for CML patients with minimal residual disease (i.e.,  $<5\%$  Ph positivity), because of the false positivity potential. In such cases, other procedures like HMF are necessary. However, PB I-FISH is a practical and less invasive monitoring procedure allowing rational treatment decisions at particular periods during the course of therapy, when Ph-positive disease is reduced, but not eliminated, below the conventional cytogenetic levels.

## 8. Conclusions and Recommendation

Peripheral blood I-FISH is most useful in assessing early treatment response of patients receiving IFN- $\alpha$  therapy STI 571 or DLI. However, the background rate of 5% false positivity limits its use to assess minimal residual disease. More sensitive quantitative methods (HMF) should be performed for that purpose. HMF recognized Ph-positive cells in 16% of patients characterized as having a complete cytogenetic response after IFN- $\alpha$  therapy and identified statistically significant differences between the frequencies of Ph-positive cells in samples that differed by  $<4\%$  during therapy. In addition, HMF was a useful quantitative technique to detect and monitor Ph-positive disease after allogeneic BMT or peripheral blood stem cell transplantation, since early detection of CML relapse post-BMT is increa-

singly important for DLI intervention.

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**Table 2.**

A Practical Monitoring Approach to Patients on Therapy.

1. Cytogenetic studies at diagnosis to detect Ph-positive disease and clonal evolution, then yearly to monitor clonal evolution.
2. On therapy:
  - a. PB I-FISH studies as long as Ph-positive disease is expected to be more than 5-10%
  - b. Shift to HMF(BM) when Ph-positive cells are  $< 5\%$  to monitor MRD
3. Value of HMF in monitoring CML course and in assessing MRD with IFN- therapy, STI571 or post BMF:
  - a. Monitor minor interval changes of % Ph-positive cells (as low as 5% changes)
  - b. May consider stopping IFN- therapy if 0 out of 1000 Ph-positive metaphases by HMF
  - c. Optimize therapy (additional agents; DLI) for significant but minor changes in disease status
  - d. Optimal timing to stop STI571 therapy based on FISH results : not yet determined.

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