Abstract. Background: Several attempts have been made to determine whether interphase fluorescence in situ hybridization (I-FISH) on bone marrow or peripheral blood specimens is a good alternative to conventional cytogenetics (CC) in calculating the residual proportion of Philadelphia (Ph) chromosome-positive cells during treatment follow-up of patients with chronic myeloid leukemia. Materials and Methods: Nineteen patients were selected for I-FISH follow-up compared to CC. All samples were also classified into 4 groups according to the percentage of residual Ph chromosome-positive metaphases analyzed in CC. I-FISH was performed using the LSI bcr/abl dual ES color probe (Vysis®). Results: A high correlation was observed between the frequency of Ph chromosome-positive cells, assessed by CC and I-FISH \(p<0.001\). A high correlation was found between CC and I-FISH for 12 patients, but not for the remaining 7. Applying the same classification for I-FISH did not show a good relationship between the two techniques \(p<0.001\). Conclusion: Dual color I-FISH is a reliable method to monitor the size of the Ph chromosome-positive clone in bone marrow of treated CML patients. However, it has to be complementary to conventional cytogenetics because it cannot detect the emergence of other chromosomal abnormalities in Ph chromosome-positive or -negative cells.

Chronic myeloid leukemia (CML) is a myeloproliferative syndrome which originates in a multipotent hematopoietic stem cell. Ninety-five % of the CML patients have a Philadelphia (Ph) chromosome. The Ph chromosome is the marker of this disease and results from the translocation \(t(9;22)(q34;q11)\) (1). In approximately 5 to 10% of the cases, there is a complex translocation with three to five chromosomes implicated with the chromosomes 9 and 22. In some cases only, a masked Ph chromosome can be found with a normal karyotype, as a result of a cryptic rearrangement (2). The Ph translocation results in the formation of two new chimeric genes, the \(5'\text{abl}-3'\text{bcr}\) gene formed on the der(9) and the \(5'\text{bcr}-3'\text{abl}\) fusion gene on the Ph chromosome.

CML treatment has dramatically improved with the introduction of interferon alfa and, more recently, of imatinib (3,4). The cytogenetic response to the treatment can be monitored by following the proportion of Ph chromosome-positive metaphases, which has been found to be very useful (3,5-7). However, interferon alfa and imatinib induce bone marrow hypoplasia in a significant number of patients. Therefore, a sufficient number of metaphases is sometimes difficult to obtain. This can lead to a less accurate assessment of the residual proportion of Ph chromosome-positive cells (3,4,8). Fluorescence in situ hybridization (FISH) can detect specific chromosomal rearrangements, not only on metaphases (M-FISH), but also on interphase cells (I-FISH). In this paper, we report our experience on 19 Ph chromosome-positive patients studied during follow-up using conventional cytogenetics (CC) and I-FISH to determine whether this latter method could be an alternative to banding cytogenetics.

Materials and Methods

Since 1995, all the CML pre-treated specimens were stored at -20°C. Briefly, the bone marrow (BM) cultures were synchronized for 17 h by fluorodeoxyoxuridine (FudR \(10^{-7}\)M), before being released by thymidine \(10^{-8}\)M for 6 h. They were then exposed to colcemid for

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15 min followed by hypotonic treatment with KCl (0.075mol/l) at 37°C for 20 min and several washes with fixative (methanol/acetic acid 3:1). R banding by heat denaturation with Earle’s solution at 87°C was used and 20 metaphases were studied per specimen (9). The karyotypes were described according to the International System for Chromosome Nomenclature (ISCN 1995) (10).

For each patient, the follow-up included a bone marrow sample when possible, and on all the specimens available during follow-up. Briefly, before hybridization, DNA slides were immersed in a jar of 2xSSC/0.4%NP40 solution for 30min at 37°C and then immediately passed through an ethanol series (70%, 85% and 100%). The denaturation was performed simultaneously on bone marrow nuclei and probes for 1min at 75°C. The slides were incubated overnight in a dark humidity chamber at 37°C. The slides were washed for 45sec in 0.4xSSC/0.1%NP40 at 72°C and 20sec in 2xSSC/0.1%NP40 at room temperature. Finally, they were counterstained with 4',6-diamidino-2-phenyl-indole (DAPI) (9).

The slides were analyzed using a Zeiss AxiosPlan Microscope (Zeiss, Le Pecq, France). Subsequent image acquisition was performed using a CCD camera with Isis (significant in situ imaging system) (MetaSystems, Altlussheim, Germany), as previously described (9).

Using BCR-ABL ES probes, normal cells display 2 red signals and 2 green signals (2R2G), and abnormal cells with the breakpoint in M-bcr display 2 red, 1 green and 1 yellow fusion signals (2R1G1Y). When the breakpoint is in m-bcr, cells display 1R1G2Y pattern. Cells with the deletion of the 5’abl region show 1 red, 1 green and 1 yellow signals (1R1G1Y). A total of 300 interphase cells were scored, as 20 metaphases for comparison purposes with the conventional method.

For the present study, 19 of the 112 CML patients thus far analyzed in the cytogenetic laboratory were selected because they presented different patterns of conventional cytogenetic evolution. For each of them, the follow-up included a bone marrow sample for cytogenetical purposes every 6 months or year, at which time a cytogenetic analysis, including banding techniques, M-FISH and I-FISH, was performed. These patients were classified into 4 groups depending upon the percentage of residual Ph chromosome-positive metaphases in conventional cytogenetics: no cytogenetic response (>90% Ph chromosome-positive cells), minor CR (35-90%), major CR (1-34%) and complete CR (0%) (11).

Spearman correlation coefficients and p values were calculated using the software SPSS. The statistical significance was set at p<0.05.

Results

The risk of false positivity is reduced to a minimum because of the presence of the extra signal. However, we still examined the bone marrow specimens from 10 patients with thrombocytopenia, in whom conventional cytogenetics showed no Ph chromosome, to determine the positivity cut-off level. Three hundred nuclei were scored for each control. The mean false positivity percentage and standard deviation was 0.37% ± 0.075%. The positivity cut-off level was set at the mean value plus 3 standard deviations, that is 0.595%.

A total of 153 BM samples from 19 CML patients was analyzed. The mean percentage of Ph chromosome-positive metaphases at diagnosis was 100%, compared with a mean percentage of 94.6% (standard deviation SD: 2.6%) in nuclei showing a fusion signal. In the other samples, 33.2% (SD: 38.5%) and 26.3% (SD: 31.3%) of the cells were found to be Ph chromosome-positive by CC and I-FISH, respectively. A high correlation was observed between the frequency of Ph chromosome-positive cells, assessed by CC and I-FISH, as shown in Figure 1 (r=0.95, p<0.001).

The results of CC and I-FISH for each specimen of 8 of the 19 CML patients are shown as examples in Figure 2. Every specimen that had a Ph chromosome-positive metaphase in CC also had cells with a fusion signal in I-FISH. Among the 55 specimens with no Ph chromosome-positive metaphase, only 4 had no fusion signal-negative cell by I-FISH. However, 9 specimens had less than 0.595%, the positivity cut-off level. Spearman correlation coefficients were calculated for each of the 19 patients. A high correlation was found between CC and I-FISH for 12 patients, but not for the remaining 7: r ranging from 0.99 to 0.35, p values ranging from 0.49 to less than 0.001. Among those 7 patients, 5 were in complete cytogenetic remission, as found by conventional cytogenetics, and one did not respond to the treatment.

All the samples, except those obtained at diagnosis, were classified into the different categories of cytogenetic response based on conventional cytogenetics. Applying the same classification for I-FISH did not show a good relationship between the two techniques (kappa=0.32; p<0.001) (Table I).

Discussion

Several groups have already tried to determine whether I-FISH on bone marrow or peripheral blood specimens was a good alternative to conventional cytogenetics in calculating the residual proportion of Ph chromosome-positive cells (12-17).
To achieve their goals, they correlated the I-FISH results with the metaphase cytogenetics using the Spearman correlation coefficient between individual samples. As far as we are aware, only one study used serial specimens from the same patient to analyze the evolution of the frequency of Ph chromosome-positive cells calculated from CC and I-FISH results over a period of 2 years maximum (15). We used a similar approach but the length of follow-up extended up to 6 years.

Conventional cytogenetics is a high cost, time-consuming technique which can fail due to the lack of or a reduced number of metaphases, as often seen during treatment with interferon and imatinib. I-FISH appears to be a good
alternative because it is quicker and more sensitive (greater number of cells analyzed). However, the first probes to be developed had a single fusion signal leading to a false positivity rate as high as 3 to 4% (12-14). Moreover, fusion signal scoring was subjective, therefore leading to false negativity (12).

The introduction of the D-FISH (Oncor®) and LSI bcr/abl ES probe (Vysis®) considerably decreased the rate of false-positive cells, ranging from 0.1% to 0.25% (15-17). This led to a positivity cut-off level ranging from 0.4% to 1.4% (15-17). Our results, based on 3000 nuclei, are in agreement with those previously reported, with a false positivity rate of 0.37%. Therefore, the use of these more recent probes for I-FISH appears to be an efficient and accurate enough method to monitor CML treatment.

However, in 10 to 15% of the CML patients, there is a deletion of the 5' abl region on the derivative chromosome 9, thus removing the extra signal (18). In those circumstances, the false positivity rate is much higher and I-FISH may not be suitable for follow-up. However, recently, Smoley et al. (19) introduced a tricolor, dual-fusion FISH method to decrease the rate of false positivity in Philadelphia chromosome-positive CML associated with a deletion on the derivative chromosome 9.

Because bone marrow aspirate is an invasive and painful procedure, several workers studied whether I-FISH on peripheral blood could be a valid alternative compared to bone marrow karyotyping or I-FISH. Some found a good correlation, but discrepancies between both methods in individual patients and between studies were found (15,16,20-22). A good correlation ($r=0.89$, $p=0.001$) was found between bone marrow karyotyping and peripheral blood FACs-sorted neutrophils (fluorescence-activated cell sorting).

A strong correlation was found between bone marrow karyotyping and I-FISH in all the studies, including ours (14,16,23). Our study over a long time period in 19 patients showed that the strong correlation found in other studies for individual samples do not always apply for individual patients, both curves being highly correlated in many, but not all, of the patients. Therefore, mixing data from both techniques for patient follow-up may be misleading. The I-FISH curve is usually below the conventional cytogenetics curve. Indeed, some nuclei without fusion signal represent normal cells, not all the cells in each hematopoietic lineage usually being involved in the neoplastic process. Furthermore, most B-lymphocytes and almost all T-lymphocytes are Ph chromosome-negative in CML (17,22,24,25).

In conclusion, dual color I-FISH is a reliable method to monitor the size of the Ph chromosome-positive clone in the bone marrow of treated CML patients. It offers the advantage over conventional cytogenetics of being more precise mostly in good responders (major or complete cytogenetic response), but the two methods are not interchangeable. However, dual color I-FISH has to be complementary to conventional cytogenetics because it cannot detect the emergence of other chromosomal abnormalities in Ph chromosome-positive or -negative cells.

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References


| Table I. Distribution of the patients in classes depending upon the rate of remaining Ph chromosome-positive cells, as found by CC and I-FISH. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Conventional cytogenetics       | >90% Ph cells   | 35-90% Ph cells | 1-34% Ph cells  | 0 Ph cell       |
| I-FISH                          | >90% Ph cells   | 3               | 18              | 0               |
|                                 | 35-90% Ph cells | 0               | 22              | 12              |
|                                 | 1-34% Ph cells  | 0               | 2               | 34              |
|                                 | 0 Ph cell       | 0               | 0               | 42              |
|                                 |                 |                 |                 | 13              |


