Chronic myeloid leukemia: a prospective comparison of interphase fluorescence in situ hybridization and chromosome banding analysis for the definition of complete cytogenetic response: a study of the GIMEMA CML WP

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In chronic myeloid leukemia, different methods are available to monitor the response to therapy: chromosome banding analysis (CBA), interphase fluorescence in situ hybridization (I-FISH), and real-time quantitative polymerase chain reaction (RT-Q-PCR). The GIMEMA CML WP (Gruppo Italiano Malattie Eematologiche Adulto Chronic Myeloid Leukemia Working Party) has performed a prospective study to compare CBA and I-FISH for the definition of complete cytogenetic response (CCgR). Samples (n = 664) were evaluated simultaneously by CBA and I-FISH. Of 537 cases in CCgR, the number of positive nuclei by I-FISH was less than 1% in 444 cases (82.7%). Of 451 cases with less than 1% positive nuclei by I-FISH, 444 (98.4%) were classified as CCgR by CBA. The major molecular response rate was significantly greater in cases with I-FISH less than 1% than in those with I-FISH 1% to 5% (66.8% vs 51.6%, P < .001) and in cases with CCgR and I-FISH less than 1% than in cases with CCgR and I-FISH 1% to 5% (66.1% vs 49.4%, P = .004). I-FISH is more sensitive than CBA and can be used to monitor CCgR. With appropriate probes, the cutoff value of I-FISH may be established at 1%. These trials are registered at http://www.clinicaltrials.gov as NCT00514488 and NCT00510926. (Blood. 2009;114:4939-4943)

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder induced by a chimeric gene that results from the fusion of the ABL gene on chromosome 9 with the BCR gene on chromosome 22, leading to the formation of a new leukemia-specific fusion gene that codes for constitutively activated protein tyrosine kinases (PTK) of different molecular weight (p210, rarely p230 or p185/190). The oncogenic PTK, which is located in the cytoplasm, is responsible of the leukemic phenotype through the constitutive activation of several downstream pathways.1-3 In more than 95% of cases, the translocation between chromosome 9 and 22 is balanced and results in the formation of a small chromosome 22 that was identified originally as Philadelphia (Ph). Rare variant translocations may be masked and detected by fluorescence in situ hybridization of interphase nuclei (I-FISH).5-7 The BCR-ABL mRNA is detected by reverse transcriptase polymerase chain reaction (PCR) and may be quantified by real-time quantitative PCR (RT-Q-PCR).6-8 After the introduction of interferon-α and the PTK inhibitor imatinib mesylate (IM), it has become more and more important to monitor cytogenetically the response to treatment and the course of the disease.9-13 On the basis of chromosome banding analysis (CBA) of marrow cell metaphases, the cytogenetic response (CgR) is classified as none, minimal, minor, partial, or complete according to the percentage of Ph+ metaphases (95%, 95%-66%, 65%-36%, 35%-1%, and none).14 The achievement and the maintenance of a complete cytogenetic response (CCgR) are of particular importance because a CCgR is the most solid, confirmed, early surrogate marker of progression-free survival and overall survival.11-16 The definition of CgR by CBA requires marrow cells, which cannot be always sampled, and an adequate number of banded metaphases, which cannot be always obtained. For these reasons, I-FISH is sometimes used, with increasing frequency, as a substitute for CBA, but although there is a fairly good relationship between I-FISH and CBA data,17-22 there are no controlled and shared...
definitions of CgR by I-FISH. This study was designed with the purpose of comparing CBA and I-FISH data for the definition of CCgR.

Methods

Patients

All the patients, at least 18 years of age, were required to have a Ph⁺ and BCR-ABL⁺ CML in early chronic phase (CP). They were enrolled in 3 prospective and concurrent studies: CML/021 (ClinTrials.gov no. NCT00514488), a phase 2 trial exploring IM 800 mg daily in intermediate Sokal risk patients with CP CML; CML/022 (ClinTrials.gov no. NCT00510926), a phase 3 trial comparing IM 400 versus 800 mg daily in high Sokal risk patients with CP CML; and CML/023, an observational study of IM 400 mg daily in patients with CP CML. These studies were promoted, sponsored, and operated by the CML Working Party of GIMEMA (previously the Italian Cooperative Study Group on CML). These studies included a prospective comparison of CBA and I-FISH data on marrow cells after 6 and 12 months of therapy. The studies were approved by the independent ethics committee of S. Orsola-Malpighi Bologna University Hospital and the ethic committees of all participating institutions and were operated according to Good Clinical Practices and the Declaration of Helsinki. Written informed consent was required and provided by all patients. The bone-marrow samples of 68 non-CML patients, who had given informed consent to cytogenetic studies for diagnostic purposes, also were analyzed by the use of I-FISH in the Bologna laboratory.

Cytogenetics: CBA

The GIMEMA CML Working Party has established a network of cytogenetic laboratories throughout Italy (supplemental Table 1, available on the Blood website; see the Supplemental Materials link at the top of the online article). In 14 of these laboratories, researchers performed CBA and FISH studies for more than one clinical center (reference laboratories), and researchers in 24 laboratories performed CBA and FISH studies only for their respective clinical center. In most laboratories, CBA and I-FISH were performed by different technicians. All studies were performed on unfractiated bone marrow cells, which were referred to the laboratories within 24 hours from sampling. CBA was performed after short-term culture (24 and/or 48 hours). The cells were treated with colchicine and hypotonic solution, then they were centrifuged, and the resulting pellet was fixed and washed in methanol/acetic acid (3:1). The cells were resuspended in fixative and dropped on slides. Karyotypes were examined after the G banding technique and described according to International System for Human Cytogenetic Nomenclature (ISCN 1995). At least 20 metaphases per sample were analyzed in 87% of samples and 10 to 19 metaphases in 13% of samples. A central review was not performed.

I-FISH analysis

I-FISH was performed on marrow cells prepared according to the technique described previously and by the use of DNA probes that hybridize to BCR and ABL regions. The probes were commercially available. All labs used BCR-ABL Extra-Signal (ES; Vysis-Abbott), Dual-Color Dual-Fusion (DCDF; Vysis-Abbott), or Dual-Fusion FISH (D-FISH; Q-Biogene-Oncor) probes. Only one laboratory used a “home-brew” PAC/BAC system: a pool of PAC, RP5-1132H12, and RP5-835J22, for ABL gene and BAC, RP11-164N13 for BCR gene. Dual-color single-fusion probes were never used in this study because they generate only one fusion signal in Ph⁺ cells, and therefore cutoff values may be remarkably high. The number of nuclei analyzed by I-FISH ranged between 166 and 2677 (median, 300; mean, 298).

Molecular studies

Qualitative reverse transcription polymerase chain reaction (PCR) for BCR-ABL transcript was routinely performed at enrollment for determining the type of transcript. Peripheral blood samples for real-time quantitative PCR (RT-Q-PCR) were collected before therapy; after 3, 6, and 12 months; and every 6 months thereafter. All samples and tests were centralized in Bologna. Whole buffy-coat cells were used. RNA extraction, reverse transcription, RT-Q-PCR were performed according to European recommendations, as previously described. RT-Q-PCR was performed on the ABI PRISM 7700 Sequence Detector (Perkin Elmer). ABL was used as a housekeeping gene to correct differences in RNA quality and/or reverse transcription efficacy. BCR-ABL and ABL plasmid dilutions (Ipsogen Inc) were used as standards, and the final results were calculated as the ratios BCR-ABL-to-ABL and expressed in percentages. All experiments were performed in duplicate, and the results were expressed as percent ratio to ABL. The BCR-ABL/ABL ratios were further multiplied by the conversion factor of the Bologna laboratory to set the results on an international scale. Samples yielding an ABL threshold cycle greater than 30, corresponding to less than 1000 ABL transcript copies, were considered as having degraded RNA and discarded. We defined major molecular response (MMoR) as a BCR-ABL/ABL ratio equal less than 0.1%.

Statistical analysis

The statistical significance of differences was assessed with the Student t test and the Mann-Whitney U test of continuous variables and with the Fisher exact test for categorical variables. The GraphPad Prism 4 (GraphPad Software Inc) was used throughout.

Results

Five hundred sixty-seven patients were studied at baseline; 515 of them (90.8%) were evaluable cytogenetically. Thirty had a variant translocation; 665 marrow samples were evaluable for CBA and I-FISH at the same time; and 614 were in complete or partial CgR (PCgR) by standard CBA-based definition and were analyzed for comparison of CBA and I-FISH data. In addition, 512 of these 614 samples (83.4%) were evaluable for BCR-ABL transcripts level at the same time and were analyzed for comparison of CBA, I-FISH, and RT-Q-PCR data. In these samples, the number of metaphases analyzed by CBA ranged between 10 and 100 (median, 20; mean, 24), and the number of nuclei analyzed for I-FISH ranged between 166 and 2677 (median, 300; mean, 298).

First, we analyzed I-FISH data according to CBA data (Table 1). There were 537 cases of CCgR by CBA, of which 444 (82.7%) had

Table 1. Distribution of I-FISH data according to CBA data

<table>
<thead>
<tr>
<th>Cytogenetic response by CBA</th>
<th>Less than 1% BCR-ABL⁺ nuclei</th>
<th>1%-5% BCR-ABL⁺ nuclei</th>
<th>More than 5% BCR-ABL⁺ nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCgR (n = 537), no Ph⁺ metaphases</td>
<td>444 (82.7)</td>
<td>71 (13.2)</td>
<td>22 (4.1)</td>
</tr>
<tr>
<td>PCgR (n = 77), 1%-35% Ph⁺ metaphases</td>
<td>7 (1.1)</td>
<td>82 (41.6)</td>
<td>38 (49.3)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

CBA indicates chromosome banding analysis; CCgR, complete cytogenetic response; I-FISH, interphase fluorescence in situ hybridization; and PCgR, partial cytogenetic response.
Discussion

Progress in treatment of Ph⁺ CML has been always measured by the degree of CgR. The degree of CgR is assessed by the use of CBA of marrow cell metaphases, based on the percentage of Ph⁺ metaphases. Although the number of banded metaphases that are conventionally required is small (n = 20), the value of the different degrees of CgR, which were established during interferon-α studies, also has been validated with IM, and achieving a CgR is still the most robust early surrogate of the outcome of therapy. The authors of several reports pointed out that the relationship between CBA and I-FISH data was significant and excellent but they did not allow one to translate the figures that define CgR with CBA data into those that should define CgR with I-FISH data. A percentage of Ph⁺ metaphases ranging between 1% and 35% defines a CgR as partial, but the same figures cannot be applied by use of the percentage of BCR-ABL⁺ nuclei. However, the 2 techniques can be concordant in the most important definition of CgR, that is, CgR, corresponding to the absence of Ph⁺ metaphases of a total of at least 20 banded metaphases.

Table 3. Distribution of MolR according to CBA and I-FISH data

<table>
<thead>
<tr>
<th>Molecular response</th>
<th>Major MolR, n (%)</th>
<th>Median BCR-ABL transcripts level*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ph⁺ metaphases and &lt; 1% BCR-ABL⁺ nuclei (n = 425)</td>
<td>281 (66.1)</td>
<td>0.035</td>
<td>.004</td>
</tr>
<tr>
<td>No Ph⁺ metaphases but ≥ 1% BCR-ABL⁺ nuclei (n = 87)</td>
<td>43 (49.4)</td>
<td>0.079</td>
<td>.06</td>
</tr>
<tr>
<td>No Ph⁺ metaphases and &lt; 1% BCR-ABL⁺ nuclei (n = 396)</td>
<td>263 (66.4)</td>
<td>0.031</td>
<td>.68</td>
</tr>
<tr>
<td>0.1%-0.9% BCR-ABL⁺ nuclei (n = 29)</td>
<td>21 (72.4)</td>
<td>0.043</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>1%-5% BCR-ABL⁺ nuclei (n = 95)</td>
<td>49 (51.6)</td>
<td>0.082</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

CBA indicates chromosome banding analysis; I-FISH, interphase fluorescence in situ hybridization; and MolR, molecular response.

*Bcr-ABL/ABL% on the international scale.
Historically, the definition of the number of BCR-ABL+ cells by I-FISH was disturbed by the use of single fusion probes, which may lead to false-positive data, and tends to overestimate the number of positive nuclei, compelling one to establish a cutoff value of 5% or more. In this study, as in all other recent studies, only Dual Fusion (DF) and ES FISH strategies were used. The DF strategy uses probes that span the common breakpoints in the ABL and BCR gene regions, generating 2 novel fusion signals caused by reciprocal t(9;22): 1 on the derivative chromosome 9 and 1 on the derivative of chromosome 22. Thus, cells with a classical t(9;22) will display a FISH pattern with 1 red (normal 9 chromosome), 1 green (normal 22 chromosome), and 2 “yellow” fusion signals (derivative 9 and 22 chromosomes with BCR-ABL and ABL-BCR fusions). The detection of 2 fusion signals can virtually exclude false-positive nuclei. With the ES FISH strategy, the Ph+ cells display 2 red (normal and derivative 9 chromosome), 1 green (normal 22 chromosome), and 1 yellow fusion signals (derivative 22 chromosome).

Using these strategies, we examined 20 400 interphase nuclei in 68 non-CML marrow samples. We found only 16 positive nuclei (0.078%) and only 3 samples (4.4%) with more than 1% positive nuclei. Table 5 lists the results of 8 independent studies, reporting the ES FISH strategy, the Ph+ cells display 2 red (normal and derivative 9 chromosome), 1 green (normal 22 chromosome), and 1 yellow fusion signals (derivative 22 chromosome).

I-FISH cannot be used to assess all the different degrees of the response, from minimal to partial; although the relationship between the percentage of Ph+ metaphases and the percentage of BCR-ABL+ nuclei may be significant, there are no data showing that the percentages are the same. However, I-FISH can be used to substitute for metaphase CBA, once all the metaphases are Ph+, that is to say once the CgR is defined as complete by CBA. The cost of the reagents is greater for I-FISH, but CBA is technically more demanding and requires specifically and well-trained technicians. Moreover, the use of I-FISH will allow monitoring the completeness of the response better than CBA if the number of metaphases is small. In this study, I-FISH was performed on marrow cells, but the authors of several studies20-22,24,26,27 have already shown that there are no differences in I-FISH results between marrow and blood samples.

Taking into account that I-FISH may be more sensitive than CBA because it also correlates better with MolR, the detection of BCR-ABL+ cells by I-FISH always requires a confirmatory test with CBA before one can conclude that a CgR has been lost. CBA also is required to identify additional chromosome abnormalities in Ph+, in case of response loss, and to identify other chromosome abnormalities in Ph+ cells, in case of hematologic abnormalities, suggesting the development of a myelodysplastic condition.

Whether I-FISH may be the preferred technique for monitoring CML patients who are in CCR is still a matter of debate and also depends on the availability and the reproducibility of the techniques for the quantification of BCR-ABL transcripts level.

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