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 Ematologiche 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 cut-off 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 constitutionally 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).[2-5] 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,[17-28] there are no controlled and shared

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

	Cytogenetic response by I-FISH, n (%)		
Cytogenetic response by CBA	Less than 1% *BCR-ABL*$^+$ nuclei	1%-5% *BCR-ABL*$^+$ nuclei	More than 5% *BCR-ABL*$^+$ nuclei
CCgR (n = 537), no Ph$^+$ metaphases	444 (82.7)	71(13.2)	22 (4.1)
PCgR (n = 77), 1%-35% Ph$^+$ metaphases	7 (9.1)	32 (41.6)	38 (49.3)
P	< .001	< .001	< .001

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

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 unfractionated 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).[29] 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.[5] 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.[30-33] RT-Q-PCR was performed on the ABI PRISM 7700 Sequence Detector (Perkin Elmer).[34] *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.[8,11,15,34] 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 (MMolR) as a BCR-ABL/ABL ratio equal less than 0.1%.[11,13,14]

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 2. Distribution of CBA data according to I-FISH data

Cytogenetic response by I-FISH	Cytogenetic response by CBA, n (%)	
	No Ph+ metaphases, CCgR	1%-35% Ph+ metaphases, PCgR
Less than 1% *BCR-ABL*+ nuclei (n = 451)	444 (98.4) $P < .001$	7 (1.6) $P < .001$
1%-5% *BCR-ABL*+ nuclei (n = 103)	71 (68.9) $P < .001$	32 (31.5) $P < .001$
More than 5% *BCR-ABL*+ nuclei (n = 60)	22 (36.7)	38 (63.3)

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

less than 1% positive nuclei by I-FISH, but 71 (13.2%) had 1% to 5% positive nuclei, and 22 (4.1%) even more than 5% positive nuclei. In contrast, there were 77 cases of PCgR by CBA, of which only 7 (9.1%) had less than 1% positive nuclei by I-FISH, whereas 32 (41.6%) had 1% to 5% positive nuclei, and 38 (49.3%) had more than 5% positive nuclei. The data show that a substantial minority (17.3% overall) of cases who were classified as CCgR by CBA had 1% or greater positive nuclei by I-FISH, whereas only few cases who were classified as PCgR by CBA had less than 1% positive nuclei by I-FISH.

Second, we analyzed CBA data according to I-FISH data (Table 2). There were 451 cases with less than 1% positive nuclei by I-FISH, of whom almost all (98.4%) had no Ph+ metaphases. In contrast, 68.9% and 36.7% of cases with 1% to 5% or more than 5% positive nuclei, respectively had no Ph+ metaphases.

Third, we compared the MolR of the patients classified as CCgR by CBA (100% Ph−) with the MolR of the cases with less than 1% positive nuclei by I-FISH. The proportion of MMolR was 63.3% in the former and 66.8% ($P = .24$) in the latter, showing no significant differences between the group defined by CBA and the group defined by I-FISH. However, the percentage of MMolR was significantly greater in the 425 cases with no Ph+ metaphases and less than 1% I-FISH–positive nuclei, than the 87 cases with no Ph+ metaphases CCgR and more than 1% I-FISH–positive nuclei (66.1% vs 49.4%, $P = .004$; Table 3).

Next, we examined the distribution of MMolR according to the number of positive nuclei by I-FISH (Table 3) There were no differences between the cases with no positive nuclei and those with 0.1% to 0.9% positive nuclei, whereas in the cases with 1% to 5% positive nuclei the percentage of MMolR was significantly lower and the BCR-ABL transcript level was significantly greater.

Table 4. Control non-CML samples

BCR-ABL+ nuclei by I-FISH	Samples, n (%)	Positive nuclei, n (%)
0 (none)	63/68 (92.6)	0/18 900
0.1%-0.9%	2/68 (2.9)	3/600 (0.5)
1%-5%	3/68 (4.4)	13/900 (1.4)
More than 5%	0/68	0/0

Positive nuclei were recorded in 5 of 68 samples, but the positivity was greater than 1% in only 3 of 68 samples.

I-FISH indicates interphase fluorescence in situ hybridization.

The total number of cases classified as MMolR was 333, of which 263 (79%) had no positive nuclei at I-FISH, 21 (6.3%) had 0.1% to 0.9% positive nuclei, and 49 (14.7%) had 1% to 5% positive nuclei. No cases of MMolR were found among the cases with more than 5% positive nuclei.

I-FISH was performed at one laboratory (Bologna) also on 68 selected *BCR-ABL*− bone-marrow samples, scoring 300 nuclei in each sample (Table 4). The percentage of positive nuclei was 1% or greater (1%, 1.3%, and 2%) in 3 samples, was less than 1% (0.3% and 0.7%) in 2 samples, and was 0 in 63 samples. The mean percentage of *BCR-ABL*+ nuclei was 0.08 (SD = 0.3). Thus a positivity cutoff value was fixed at 1% (mean + 3 SD).

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.[9-13] Although the number of banded metaphases that are conventionally required is small (n = 20),[13,14] the value of the different degrees of CgR, which were established during interferon-α studies, also has been validated with IM, and achieving a CCgR is still the most robust early surrogate of the outcome of therapy.[11-16] The authors of several reports[17-28] 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, CCgR, 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

		Molecular response			
		Major MolR, n (%)	*P*	Median BCR-ABL transcripts level*	*P*
Cytogenetic response by I-FISH and by CBA	No Ph+ metaphases and < 1% *BCR-ABL*+ nuclei (n = 425)	281 (66.1)	.004	0.035	.06
	No Ph+ metaphases but ≥ 1% *BCR-ABL*+ nuclei (n = 87)	43 (49.4)		0.079	
Cytogenetic response by I-FISH	No *BCR-ABL*+ nuclei (n = 396)	263 (66.4)	.68	0.031	.43
	0.1%-0.9% *BCR-ABL*+ nuclei (n = 29)	21 (72.4)	< .001	0.043	< .001
	1%-5% *BCR-ABL*+ nuclei (n = 95)	49 (51.6)		0.082	

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

*BCR-ABL/ABL% on the international scale.

Table 5. Cutoff values for "false *BCR-ABL*$^+$ cells," as reported in previous studies, by the use of I-FISH

References	Probe used	Cutoff	Samples
Dewald et al, 1998[19]	D-FISH	Not identified*	35 normal subjects
Buño et al, 1998[20]	D-FISH	0.8%	PBL from 10 patients without any malignant hematologic disorder and BM from 10 normal bone marrow transplant donors
Chomel et al, 2000[9]	D-FISH	1%†	PBL or BM from 5 patients with hematologic disorders other than CML
Le Gouill et al, 2000[22]	ES	1.2%†	14 BM from AML or *BCR/ABL*$^-$ myeloproliferative syndrome patients and 10 PBL from healthy donors
Chase et al, 2000[35]	DCDF	1%‡	Not specified
Primo et al, 2003[36]	ES	0.9%§	20 *BCR/ABL*$^-$ BM samples
Raanani et al, 2004[26]	ES	1%‖	Not specified
El-Zimaity et al, 2004[37]	ES	1.2%†	20 normal control samples

Landstrom et al,[27] in a study of Ph$^+$ CML, used a cutoff of 0.6%, citing Dewald et al.[19]

AML indicates acute myeloid leukemia; D-FISH, dual FISH (from Q-Biogene); DCDF, dual-color dual-fusion (from Vysis-Abbott); ES, extra-signal FISH (from Vysis-Abbott); BM, bone marrow; PBL, peripheral blood.

*"Based on 200 nuclei, the mean percentage of false-positive cells was 0.25 ± 0.39. The normal cutoff for 6000 nuclei (for each of 3 normal specimens) was 0.079%."

†Mean + 3 SD.

‡"Although in experienced hands with good quality preparations the technique can give a very low false positive rate of 0.1%-0.2%, in practice we prefer to use cutoff rate of 1% because accuracy is seriously compromised by several factors, including slide quality and operator experience."

§Mean + 2SD.

‖"Because the FISH (ES) method has false positive rate of 1%, results were considered negative when < 1% of interphases nuclei were positive for BCR-ABL double-labeled probes."

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,[9,19,20,22,24-27,35-37] 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 *BCR/ABL* positivity detected by DF or ES FISH in non-CML samples.[9,19,20,22,26,35-37] The cutoff values that were proposed by these studies ranged between 0.8% and 1.2% (median, 1%). Therefore, we conclude that a cutoff value of 1% is fairly reasonable, even conservative, and may prevent any overestimate of the completeness of the response.

In this prospective comparative study of CBA versus I-FISH, almost all the cases (98.4%) below the cutoff value of 1% were CCgR by CBA. On the opposite, 13.2% and 4.1% of the samples that were defined as CCgR by CBA had 1% to 5% and more than 5% positive nuclei, respectively. These data suggest that I-FISH may be more sensitive than CBA for the assessment of minimal residual disease, which is not surprising, considering that more cells are examined with I-FISH than with CBA. As a matter of fact, the rate of MMolR was greater and the BCR/ABL transcripts level was lower in the patients who were negative by I-FISH and CBA that in those who were negative only by CBA (Table 3).

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 studies[20-22,24,26,27] 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 CCgR 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 CCgR 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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Authorship

Contribution: N.T. coordinated all cytogenetics studies with the contribution of G. Marzocchi, S.L., C.B., M.S., M.N., G.R.-C., E.G., U.G., E.A., S.K., M.G.G., A.G., B.C., C.C., P.B., A.C., F.A., G.F., and A.Z; M.A. coordinated the molecular studies; G.R. coordinated the clinical studies with the contribution of G. Martinelli and F.P.; and N.T. and M.B. analyzed the data and wrote the report, which was reviewed by all centers.

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For a complete list of GIMEMA CML WP participants, see the supplemental Appendix, available on the *Blood* website.

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