Detection of Chimeric BCR-ABL Genes on Bone Marrow Samples and Blood Smears in Chronic Myeloid and Acute Lymphoblastic Leukemia by In Situ Hybridization

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The presence of BCR-ABL fusion genes has important diagnostic and prognostic implications in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). The CML-specific chimeric BCR-ABL gene with a break involving the major breakpoint cluster region (M-bcr) of the BCR gene has been detected by means of fluorescence in situ hybridization (FISH). In this study, we present a FISH protocol that allows the detection of breaks in both the major and the minor breakpoint cluster region (m-bcr). Three hybridization signals of D107F9, a yeast artificial chromosome (YAC)-derived probe spanning the breakpoint regions of the BCR gene, were indicative of the translocation events. To increase the specificity further, this probe was combined with cos-abl 8, a cosmide probe flanking the breakpoint within the ABL gene for dual-color hybridization. Samples of 21 patients with CML, the ALL-derived cell line SUP-B15, and of seven patients with Philadelphia chromosome (Ph')-positive ALL (three of them with breakpoints within m-bcr) were examined. BCR-ABL fusion was detected in all cases with high specificity (false-positive nuclei: mean, 0.1%). On cytogenetic preparations, the percentages of BCR-ABL-positive interphase cells ranged from 53% to 91%. Comparable efficiencies were achieved on blood smears. In conclusion, hybridization with D107F9 and cos-abl 8 allows unambiguous diagnosis of BCR-ABL genes and is likely to become an important tool for the monitoring of therapies in patients with CML and ALL.

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THE PHILADELPHIA chromosome (Ph') is the derivative of a translocation between the long arms of chromosome 9 and 22 (t(9;22)(q34;q11)). This chromosomal abnormality was discovered in chronic myeloid leukemia (CML), where it is found in more than 90% cases. In adult acute lymphoblastic leukemia (ALL), the Ph' has been found in 17% to 30% of cases using G-banding analysis. It is the single most common chromosomal abnormality in this disease. Further importance is added to the t(9;22) by its association with poor prognosis in ALL.

In ALL, karyotype analysis using banding techniques fails in 10% to 20% of cases due to inadequate chromosome morphology or a lack of metaphases. In another 30% to 40%, only normal metaphases are found. In these cases, ALL blasts may not have been investigated. Therefore, other methods that do not depend on the preparation of metaphase chromosomes and that facilitate a rapid detection of the Ph' are of substantial interest for clinicians treating patients with Ph'-positive leukemias.

On the molecular level, the t(9;22)(q34;q11) results in the fusion of part of the ABL proto-oncogene on chromosome 9q with part of the BCR gene on chromosome 22. The breakpoints within the ABL gene are scattered over more than 175 kb upstream of exon II. In contrast, the breaks on chromosome 22 are clustered within two areas of the BCR gene, termed the major breakpoint cluster region (M-bcr) and the minor breakpoint cluster region (m-bcr). Whereas the breakpoints in CML are restricted to the M-bcr, either region is involved in ALL.

So far, the feasibility of fluorescence in situ hybridization (FISH) for the detection of the BCR-ABL fusion in interphase cells of CML-cases (M-bcr) has been demonstrated in small numbers of patients. In this report, we present data from hybridization experiments using the yeast artificial chromosome (YAC) probe D107F9 spanning the breakpoint regions in the BCR gene and cos-abl 8, a cosmide probe flanking the breakpoints on chromosome 9. With this probe set, breaks both within the M-bcr and the m-bcr are detected, and thus it is suitable for interphase diagnosis of the BCR-ABL fusion in ALL. Methanol/acetic acid–fixed bone marrow cells on cytogenetic preparations and cells on blood smears were examined.

MATERIALS AND METHODS

Patients

Six controls, 21 patients with Ph'-positive CML (no. 1 through 21; age range, 28 to 65 years; median, 45 years; nine males and 12 females), and seven patients with Ph'-positive ALL (no. 22 through 28; age range, 22 to 50 years; median, 29 years; four males and three females) were examined. G-banding analysis was performed in all but one patient (no. 23). In patients no. 24, 25, and 26, respectively. All other patients had 100% Ph'-positive metaphases; in patients no. 12 and 13, an additional Ph' was found in all metaphases. In five of seven ALL patients, the breakpoint was located using the polymerase chain reaction (PCR); in three, it was within the m-bcr (no. 23, 26, and 28) and in two patients it was within the M-bcr (no. 24 and 27). The ALL-derived cell line SUP-B15 (known to contain a breakpoint within m-bcr) was kindly provided by Dr Steve Smith, Chicago, IL) was used as a positive control.

DNA Probes

The YAC clone D107F9 (kindly provided by Dr Thomas Cremer, Heidelberg, Germany, and Dr Harold Riethman, St Louis, MO) con-

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contains a 215-kb fragment spanning the BCR gene. Before labeling, human sequences were generated from the YAC clone by a PCR protocol using primers directed against Alu sequences as described. Briefly, approximately 140 ng of genomic YAC clone DNA was added to three Alu-PCR reactions using the primers C11, C12, or both, respectively: C11 = 5'-TCC CAA AGT GCT GGG ATT ACA G3'; C12 = 5'-CTG CAC TCC AGC CTG G3'.

After initial denaturation at 96°C for 10 minutes, 30 cycles of PCR were performed with denaturation at 94°C for 3 minutes, annealing at 58°C for 1 minute, and extension at 72°C for 6 minutes. The last cycle was concluded with an extension at 72°C for 10 minutes. The products of all three reactions were combined for FISH. For cohybridization, the cosmid clone cos-ab1 8 (kindly provided by Dr Nora Heisterkamp, Los Angeles, CA) was used. This clone contains a 40-kb fragment representing 3' coding and 3' flanking sequences of the human ABL gene on chromosome 9.

Slide Preparation

Slides prepared from methanol/acetic acid–fixed bone marrow cell pellets were stored at −20°C for up to 3 years. In our hands, treatment of the slides with 100 mmol/L CaCl₂ seemed to improve the hybridization efficiency. Therefore, CaCl₂ was used as described below, before pepsin digestion and after denaturation of the specimens.

After thawing, the slides were treated with 100 mmol/L CaCl₂ (20 minutes on ice) followed by incubation with pepsin (Serva, Heidelberg, Germany) for 15 minutes at 37°C (1 mg pepsin in 100 mL 0.01 mol/L HCl). After postfixation in 0.5% paraformaldehyde (Sigma) 0.05% MgCl₂ in phosphate-buffered saline (PBS), the slides were dehydrated in a series of alcohol (70%, 90%, and 100%, 8 minutes each at room temperature) and air-dried.

Stained blood smears had been stored for up to 2 years at room temperature in the hematology laboratory. They were pretreated as described with methanol/acetic acid 19:1 and 3:1, 10 minutes each at room temperature followed by dehydration in alcohol. Slides older than 2 weeks were rehydrated in PBS and TRIS/EDTA buffer (24 hours at 37°C each) before pretreatment with methanol/acetic acid.

In Situ Hybridization

The probes were labeled by nick translation with either biotin-16-dUTP (D107F9) or digoxigenin-11-dUTP (cos-ab1 8) (Boehringer Mannheim, Mannheim, Germany). Hybridization and detection of D107F9 and cos-ab1 8 were performed as described previously, with the following modifications: for the single-color experiments, 100 to 300 ng of labeled D107F9 was used after preannealing with 100 μg of an unleaved Cot-1 DNA fraction (BRL/Life-Technologies, Gaithersburg, MD); for the dual-color hybridization, 40 to 100 ng of labeled cos-ab1 8 was added to the probe mixture. Nuclei on the slides were denatured in 70% formamide, 2× standard saline citrate, at 71°C for 150 seconds, incubated in 100 mmol/L CaCl₂ on ice for 20 minutes, and then dehydrated in ice-cold ethanol. Detection was performed with Cy3-conjugated streptavidin and fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin (Boehringer Mannheim). The signals were amplified once as described elsewhere.

Results

Single-Color FISH

The YAC-probe D107F9 spans the breakpoint cluster regions within the BCR gene on chromosome 22. Instead of two hybridization signals resulting from the normal homologs of chromosome 22, three signals are detected after hybridization to BCR-ABL–positive cells: one originates from the normal chromosome 22, one from the Ph, and one from translocated sequences of the BCR gene on the 9q + chromosome (Fig 1A and B).

Controls. D107F9 showed bright hybridization signals on metaphase and interphase cells of all controls, and at least 95% of interphase nuclei showed two hybridization signals (mean ± SD, 95.4% ± 0.5%) (Fig 2A). A mean of 2.2% (range, 0.5% to 3%; SD, 0.8%) of the nuclei had three signals (see Discussion). One signal was observed in a mean of
Fig 2.
from the statistical evaluation of the controls: the mean tients with CML (1-13), and tion on six controls (A-F), 13 pa-

The cutoff level was obtained from the statistical evaluation of the controls: the mean ± 3 SD of false-positive nuclei is consid-

tered the cutoff level for diagnosing the BCR-ABL fusion. Nuclei exhibiting three hybridization signals (B); nuclei exhibiting four hybridization signals (C).

1.5% (SD, 0.9%) of cells. The results of the single-color hybridizations from the controls, as well as from CML and ALL cases, are illustrated in Fig 3.

CML patients. BCR-ABL fusion was diagnosed if greater than 4.6% of nuclei exhibited three signals (mean ± 3 SD of the controls). In 13 patients with CML, single-color FISH was performed on methanol/acetic acid-fixed cells of cyto-
genetic preparations. Eleven patients had a single Ph' (no. 1 through 11). The percentages of nuclei exhibiting three hybridization signals ranged from 77% to 88% (mean ± SD, 83.3 ± 4.2%) (Fig 2B) in these cases. On G-banding, patients no. 12 and 13 had an additional Ph'. With FISH, three signals were seen in 19% and 41.5%, and four signals were detected in 66.5% and 44%, respectively.

ALL patients. To establish whether D107F9 also spans the m-bcr of the BCR gene, we first performed FISH on the cell line SUP-B15. In the majority of cells (75%), three signals were seen (Fig 2E). Two signals were detected in 17% of nuclei. Subsequently, seven patients with Ph'-positive ALL were examined. The percentages of cells with three hybridization signals ranged from 52.5% to 79.5% (mean ± SD, 68.3% ± 9.9%). Interestingly, the three patients who showed approximately 50% Ph'-positive metaphases by G- banding analysis (no. 24 through 26) did not differ signifi-
cantly from the ALL patients with 100% Ph'-positive meta-

phases regarding the frequency of three interphase signals (see Figs 3 and 5). This discrepancy can be explained by selection processes resulting in an increase of Ph'-negative cells in the metaphase fraction. This was confirmed by the FISH analysis of patient no. 26, in whom the number of Ph'-positive metaphase cells was similar (seven of 15 showed three signals) to the results obtained by G-banding.

Dual-Color FISH Using D107F9 and cos-ab1 8

To increase the specificity of our detection system further, we performed dual-color FISH combining D107F9 and cos- ab1 8. Using this approach, cells were only considered to be BCR-ABL—positive if they exhibited three D107F9 signals and if one of these signals colocalized with one cos-ab1 8 signal as the result from the juxtaposition of the complemen-
tary sequences on the Ph' (Fig 1C and D). In the controls (n = 5, A-E), only 0% to 0.5% of nuclei (mean ± SD, 0.1%
± 0.3%) exhibited three YAC signals and a colocalization of D107F9 and cos-abl 8. As shown in Fig 4, the percentage of false-positive nuclei was drastically reduced in comparison to evaluating either by the presence of three D107F9 signals (data see above) or by the presence of the colocalization (mean ± SD, 2.7% ± 1.4%). Data from the dual-color experiments of the controls and of both the CML and ALL samples are illustrated in Fig 5.

CML patients. Cytogenetic preparations of eight patients with CML were examined (no. 1, 5, 6, 17, 18, 19, 20, 21). As shown by G-banding, all contained a single Ph1. The percentages of nuclei with three D107F9 signals and colocalization ranged from 72.5% to 91% (mean ± SD, 78.6% ± 6.2%). In one patient (no. 1), 14% of nuclei exhibited four D107F9 signals and two colocalizations indicating the presence of a subclone with two Ph1.

In six patients with Ph1-positive CML (no. 14, 15, 16, 17, 18, 19), blood smears were examined. A mean of 79.4% (SD, 11.5%) of cells fulfilled the criteria for a BCR-ABL fusion (Fig 2G). In one patient (no. 16), 22% of nuclei with four D107F9 signals and two colocalizations were seen.

ALL patients. Seven ALL patients were examined with dual-color FISH. The majority of nuclei had three D107F9 signals and a colocalization of D107F9 and cos-abl 8 (range, 71% to 91%; mean ± SD, 82% ± 7.8%) (Fig 2F). In patients 25 and 27, who on single-color FISH had low percentages of cells with four hybridization signals, no cells were detected exhibiting four D107F9 signals and two colocalizations.
**DISCUSSION**

In this study, \( BCR-ABL \) fusion was detected by FISH with an Alu-PCR-amplified YAC probe in 28 patients with CML or Ph\(^{1}\)-positive ALL. In the single-color experiments using D107F9, 77% to 88% of cells in CML samples and 53% to 80% of cells in ALL samples were determined to carry the \( BCR-ABL \) fusion.

In previously published reports, cosmids probes flanking the breakpoints distal on the \( ABL \) gene and proximal to the \( M-bcr \) of the \( BCR \) gene were used for the detection of the \( BCR-ABL \) fusion gene in interphase cells of CML samples.\(^8,10\) Recently, another group used probe pools containing DNA fragments from microdissected chromosomes for the analysis of the \( Ph^{1} \) in CML.\(^3\) However, the signal intensities of these probes were not sufficient for interphase analysis.

Our protocol allows the detection of breakpoints both within the \( M-bcr \) and the \( m-bcr \) of the \( BCR \) gene in metaphase and interphase cells. With D107F9, screening of ALL samples for the \( BCR-ABL \) fusion by FISH becomes possible. This is of high clinical relevance, because \( BCR-ABL \)-positive ALL patients (up to 30% of all cases)\(^6,9\) have a particularly bad prognosis, which has resulted in the design of treatment protocols that stratify patients according to their \( BCR-ABL \) status.\(^22\)

Based on metaphase analysis in untreated patients with Ph\(^{1}\)-positive CML, it has been assumed that close to 100% of the nucleated cells in the bone marrow and peripheral blood are Ph\(^{1}\)-positive.\(^23\) However, only 77% to 88% of interphase nuclei in the CML patients exhibited three hybridization spots. Between 6.5% and 18.5% of nuclei with two hybridization signals were seen. Similarly, in the Ph\(^{1}\)-positive cell line SUP-B15, three signals were seen in 75% of interphase nuclei and two signals in 17%. Although this may in part be explained by incomplete hybridization of a fraction of nuclei leading to an underestimation of the number of a specific target present in the interphase nuclei (false-negatives), it may well reflect the presence of Ph\(^{1}\)-negative clones. However, in all of the CML and ALL cases examined, the percentages of \( BCR-ABL \)-positive cells were well above the cutoff level for positive samples. Thus, unambiguous identification of a Ph\(^{1}\)-positive leukemia is not hampered by the presence of false-negative cells.

Of greater concern for clinical application is the diagnosis of false-positive cells due to artifactual fluorescent spots that are counted as true signals. In some cases, decondensation of the D107F9 signals similar to that occasionally observed with alphoid probes\(^24\) was seen. Percentages of interphase cells, which were not assessable, ranged from 0% to 11.5% (mean, 4.6%;). In two patients with a single Ph\(^{1}\) on G-banding analysis (no. 1 and 9), 5% and 4.5% of interphase nuclei with four signals were seen. This is close to the cutoff level for the diagnosis of the Ph\(^{1}\) in cases with three signals (see Fig 3) and may be explained by an artifact possibly caused by decondensation of the target sequences. However, it cannot be excluded that the additional signals comprise additional target sequences (ie, a second Ph\(^{1}\)). These data clearly show the need to increase the specificity of the detection system.

This was achieved by combining D107F9 with cos-abl 8 for a dual-color hybridization experiment. Cells were only considered to be \( BCR-ABL \)-positive if they fulfilled two criteria: three signals of D107F9 and colocalization of one D107F9 and one cos-abl 8 signal. The percentage of false-positives in the controls was decreased to 0% to 0.5% using dual-color hybridization (mean, 0.1%; see Fig 4). Interestingly, in patient no. 1, again there was a significant percentage of nuclei with four D107F9 signals and also there were two colocalizations with cos-abl 8 in 14% of cells, which clearly proves the existence of a subclone with two Ph\(^{1}\). The same phenomenon was observed in blood smears of another CML patient with a single Ph\(^{1}\) on G-banding (ne. 16; 35 metaphases analyzed). In this sample, 22% of the cells exhibited four D107F9 signals and two colocalizations. This indicates that in some cases of CML, clonal evolution (additional Ph\(^{1}\)) may occur, before it can be detected by metaphase analysis.

In comparison to other methods detecting the \( BCR-ABL \) fusion (G-banding analysis, PCR, Southern blot analysis including pulse-field gel electrophoresis), FISH using our protocol with D107F9 and cos-abl 8 has several advantages. Due to its potential to examine interphase cells, the \( BCR-ABL \) fusion can be detected on the single-cell level. This is particularly useful, as the examination of blood smears is possible. For in situ hybridization on blood smears, probes with a high hybridization efficiency and strong signal intensities are required. The feasibility has been shown recently for the detection of numerical abnormalities using chromosome-specific repetitive probes\(^19,25\) and of a gene deletion using a probe pool comprising 200 kb.\(^25\) We here demonstrate the detection of the translocation t(9;22)(q34;q11) by FISH. Cell morphology and \( BCR-ABL \) status of single cells can be correlated. This allows the analysis of \( BCR-ABL \) involvement of various cell lineages, especially in Ph\(^{1}\)-positive ALL.

A quantitative assessment of the Ph\(^{1}\)-positive cell fraction can be achieved by FISH more accurately than with other molecular methods. This may play an important role in the monitoring of therapy in Ph\(^{1}\)-positive diseases. Recently, FISH to metaphase chromosomes was used to assess the remission status in patients with CML receiving interferon.\(^21\) With our protocol, similar analyses can be performed on the interphase level. To detect low percentages of residual cells in Ph\(^{1}\)-positive leukemias, FISH could be performed on cell fractions enriched for the malignant clone. In conclusion, hybridization with D107F9 and cos-abl 8 allows unambiguous diagnosis of \( BCR-ABL \) genes in interphase nuclei of clinical samples and is likely to become an important tool for the monitoring of therapies in patients with CML and ALL.

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**REFERENCES**


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