Application of Chromosome Microdissection Probes for Elucidation of \textit{BCR-ABL} Fusion and Variant Philadelphia Chromosome Translocations in Chronic Myelogenous Leukemia

By J. Zhang, P. Meltzer, R. Jenkins, X.-Y. Guan, and J. Trent

Fluorescence in situ hybridization (FISH) has become an increasingly important method for assessing chromosome rearrangement. The reciprocal translocation constituting the Philadelphia (Ph) chromosome (t(9;22)(q34;q11)) characterizes more than 90% of patients with chronic myelogenous leukemia (CML). However, in the remaining cases the Ph chromosome (genetically characterized by the fusion of the \textit{BCR-ABL} genes) is thought to arise through complex translocations that are often not readily apparent using routine chromosome-banding analysis. For this reason we have developed unique band-specific probes for two-color FISH that detect unequivocally the Ph chromosome, and its derivatives. Results of the application of these probes are illustrated by analysis of 11 cases of CML (9 of which contain “variant” translocations). The probes were generated by chromosome microdissection and in vitro amplification of the bands involved in the Ph translocation, leading to an extremely fast and sensitive approach to identify this alteration in leukemic cell populations.

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\section*{MATERIALS AND METHODS}

\textbf{Patient population.} Bone marrow (BM) samples were obtained from 11 patients with CML in various phases of their disease (Table 1) at the time of trephine biopsy for pathologic and clinical cytogenetic diagnosis. Cytogenetic analysis was performed as previously described.\textsuperscript{21}

Chromosome microdissection and in vitro amplification of dissected DNA. The procedure for chromosome microdissection is modified from Meltzer et al.\textsuperscript{26} Briefly, metaphase cells were prepared on glass coverslips, stained by trypsin-Giemsa banding, and microdissection was performed with glass microneedles controlled by a micromanipulator attached to an inverted microscope. The dissected chromosome fragments (in this case from \texttt{9q34} \textendash qrter and \texttt{22q11}) were transferred to a 5-\mu L collection drop (containing 40 mg/ml Tris-HCl, pH 7.5, 10 mg/ml MgCl\textsubscript{2}, 50 mg/ml NaCl, 200 \mu M of each deoxynucleotide triphosphate [dNTP]) and 5 pmol of a “universal primer” ([CCGACTCGAGNNNNNNAATGTTGG]\textsuperscript{26}). After dissecting the desired number of copies (15 to 20), the collection drop was covered with a drop of mineral oil and incubated at 94°C for 5 minutes. Then, an initial six cycles of

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PCR (denaturation at 94°C for 1 minute, annealing at 30°C for 1 minute, and extension at 72°C for 3 minutes) was conducted by adding approximately 0.3 U of T7 DNA polymerase (Sequenase version 2.0; USB, Cleveland, OH) at each cycle (Sequenase [1.6 U/µL] was added to the reaction mixture. The cycling conditions were those previously described26 (by sequential washing in an ethanol series and phosphate buffered saline [PBS]) before in situ hybridization.

### Table 1. Summary of Ph-Producing Translocations in 11 Patients With CML

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date Sample Received</th>
<th>Reason for Request</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3/28/91</td>
<td>New leukemia</td>
<td>46,XY, t(3;17;9;22)(q26.2;q21;q34;9q11)</td>
</tr>
<tr>
<td>2</td>
<td>2/09/88</td>
<td>CML, pre BMT</td>
<td>46,XY, t(14;22)(q32;9q11)</td>
</tr>
<tr>
<td>3</td>
<td>7/06/89</td>
<td>Leukemia, angina</td>
<td>46,XY, t(9;22;10)(q34;9q11;9q22)</td>
</tr>
<tr>
<td>4</td>
<td>2/24/90</td>
<td>CML, suspect blast crisis</td>
<td>46,XX, t(9;22;12)(q34;9q11;9q24.1)</td>
</tr>
<tr>
<td>5</td>
<td>1/29/90</td>
<td>CML</td>
<td>46,XX, t(11;21;22)(q11;9q11;9q13)</td>
</tr>
<tr>
<td>6</td>
<td>6/06/89</td>
<td>Leukemia</td>
<td>46,XX, t(7;9;22)(q12;9q34;9q11)</td>
</tr>
<tr>
<td>7</td>
<td>4/19/89</td>
<td>Leukocytosis, thrombocytosis</td>
<td>46,XY, t(4;9;22)(q31;34;9q11)</td>
</tr>
<tr>
<td>8</td>
<td>9/01/90</td>
<td>r/o CML</td>
<td>46,XX, t(1;22)(q36;9q11)</td>
</tr>
<tr>
<td>9</td>
<td>8/31/88</td>
<td>CML, preautologous BMT</td>
<td>46,XX, t(21;22)(q22;9q11)</td>
</tr>
<tr>
<td>10</td>
<td>12/17/91</td>
<td>CML</td>
<td>46,XX, t(9;22;34)(9q11)</td>
</tr>
<tr>
<td>11</td>
<td>12/18/91</td>
<td>CML</td>
<td>46,XX, t(9;22)(q34;9q11)</td>
</tr>
</tbody>
</table>

Abbreviation: BMT, bone marrow transplantation.

Karyotyping of each case was accomplished by banding analysis (G-banding and quinacrine staining) of BM samples.

* Presumed simple variant translocations with no involvement of 9q34 observed by conventional cytogenetic analysis.

† No apparent involvement of either 9q34 or 22q11 by conventional cytogenetic analysis.

### Table 2. Summary of Dual-Color FISH Analysis of 11 Patients With CML

<table>
<thead>
<tr>
<th>Case</th>
<th>Ph-Producing Translocation</th>
<th>Abnormal Chromosomes Detected by Probes</th>
<th>No. of Metaphase Cells Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>t(3;17;9;22)(q26.2;q21;9q34;9q11)</td>
<td>Ph.+der(9) Ph.+der(3)</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>t(14;9;22)(q32;9q11;9q11)</td>
<td>Ph.+der(9) Ph.+der(14)</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>t(9;22;10)(q34;9q11;9q22)</td>
<td>Ph.+der(9) Ph.+der(10)</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>t(9;22;12)(q34;9q11;9q24)</td>
<td>Ph.+der(9) Ph.+der(12)</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>t(11;21;9;22)(q11;9q11;9q34;9q15)</td>
<td>der(9) —</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>t(7;9;22)(q13;9q34;9q11)</td>
<td>Ph.+der(9) Ph.+der(7)</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>t(4;9;22)(q31;9q34;9q11)</td>
<td>Ph.+der(9) Ph.+der(4)</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>t(1;9;22)(q34;9q34;9q11)</td>
<td>Ph.+der(9) Ph.+der(1)</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>t(21;9;22)(q22;9q34;9q11)</td>
<td>Ph.+der(9) Ph.+der(21)</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>t(9;22)(q34;9q11)</td>
<td>Ph.+9q1 Ph.+9q1</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>t(9;22)(q34;9q11)</td>
<td>Ph.+9q1 Ph.+9q1</td>
<td>30</td>
</tr>
</tbody>
</table>

* Correct karyotype of Ph-producing translocation was only obtained by dual-color FISH analysis.
RESULTS

Karyotypes of CML cells. The karyotypes of leukemic cells from 11 CML patients are summarized in Table 1. Among these 11 previously undescribed patients, 2 had classic Ph+ CML, and the remaining 9 patients had complex translocations based on detailed banding analysis. Pictorial documentation of Q- or G-banded complex chromosome alterations from cases 2, 3, 5, 7, and 9 is presented in Fig 1.

Chromosomal in situ hybridization. Figure 2A provides pictorial documentation of the microdissected regions on chromosomes 9 and 22. The Micro-FISH probes provided sufficient sequence complexity to entirely encompass the BCR-ABL fusion region in all cases. The probe for 9q34 (the ABL probe) was biotin-labeled and detected with the green fluorochrome FITC. The probe for 22q11 (the BCR probe) was labeled by incorporation of a fluorochrome-labeled nucleotide (Spectrum-Orange Imagenetics), which was directly detected as red/orange fluorescence. Hybridization of both probes could be observed simultaneously with a fluorescence microscope equipped with a dual-bandpass filter.

Figure 2B illustrates the application of this Micro-FISH assay to a normal metaphase cell. Hybridization of the 9q34-ABL (green) or 22q11-BCR (red/orange) probes resulted in the identification of signals encompassing both normal copies of 9q34 and 21q11, respectively. Therefore, in situ hybridization using these band-specific probes results in the identification of two green and two red/orange signals per normal metaphase. In contrast, Fig 2, C and D, illustrates the result of dual-color hybridization to Ph+ CML cells, which shows a strikingly different hybridization pattern. Specifically, although the normal 9 and 22 stain green and red/orange, respectively, the Ph and 9q+ derivative translocation chromosomes that juxtapose the red/orange and green signals, appear yellow. The most distinctive pat-
Fig 2. (A) Chromosome microdissection of G-banded normal human chromosomes 9 and 22. Sequential photographs illustrating the microdissection of chromosome bands 9q34 (1 through 3) and 22q11 (4 through 6), respectively. (B) Representative normal metaphase painted with the 9q34 (green) and 22q11 (red/orange) Micro-FISH painting probes. Results show two green signals on the chromosome 9 homologues, and two red/orange signals on the chromosome 22 pair. (C) Hybridization of the 9q34 and 22q11 microdissection painting probes to chromosomes of case 10 exhibiting a t(9;22)(q34;q11). Results showed a single green and red/orange signal on the normal chromosome 9 and 22, and combined red/green (yellow) signals on the Ph and the 9q+ chromosomes. A triple-bandpass filter allowed the visualization of all chromosomes by a DAPI counter-stain. (D) G-banded ideogram illustrating the regions involved in probe hybridization in this reciprocal translocation.
tern that is unequivocally recognized is the reduction from two to only one domain of red/orange fluorescence (associated with the presence of only one normal chromosome 22). Thus, in cells displaying the Ph chromosome, all cells display one red/orange, one green, and two green/red/orange fusion signals. In every example of Ph\(^+\) CML examined to date, the identification of the Ph chromosome could be made definitively in 100% of metaphases examined. Significantly, this approach was successful even in metaphase preparations of very poor technical quality.

The hybridization results using the 9q34 and 22q11 probes for nine cases of CML with variant translocations were also performed with results for all cases shown in Table 2. Figure 3 presents a representative example of a complex
three-way translocation from case 8, thought by G-banding analysis to involve chromosomes 1 and 22. As illustrated, the involvement of chromosome 9 can be clearly documented by two-color FISH. Likewise, in three additional cases chromosomal alterations involving 9q34, unrecognized in band karyotypes, were readily distinguished with our Micro-FISH system (Table 2; cases 2, 5, and 9). In one patient (case 5), a three-way translocation thought to involve chromosomes 11, 21, and 22 was observed by conventional cytogenetic analysis (Table 1). Molecular analysis by PCR and Southern blotting (data not shown) documented the BCR/ABL fusion. In this case, G-banding was suggestive of the involvement of chromosome 9, but the banding pattern failed to definitively identify the involvement of 9q34 (Fig 1). Using the 9q34 probe it was possible to unequivocally recognize the involvement of chromosome 9 in this translocation by a reduction in fluorescence intensity on one homologue. This reduction of signal intensity on one chromosome 9 associated with a 9q34 translocation is similar to the involvement of chromosome 9 recognized in case 8 (Fig 3).

**DISCUSSION**

The present in situ hybridization study unequivocally documents the utility of this new Micro-FISH assay in delineating the translocation of ABL to BCR in both classic Ph+ CML patients, and patients with variant translocations. The results extended routine cytogenetic analysis in three cases (Table 2) where apparently simple translocations were in fact identified as complex translocations involving 9q34 by two-color FISH. The combination of our current report with the results of other investigators using either isotopic or nonisotopic in situ hybridization further illustrates that the involvement of chromosome 9 is obligatory in variant Ph translocations.

As mentioned previously, two-color FISH has very recently been applied by Tkachuk et al to the examination of both metaphase chromosomes and interphase nuclei in patients with CML. This approach was based on the simultaneous hybridization of ABL and BCR cosmid probes that localize to only one side of the translocation breakpoint. Accordingly, this method is ideal for identification of juxtaposed ABL/BCR sequences, but would not be expected to be useful in defining all derivative products of complex three-way or four-way rearrangements. In contrast, the Micro-FISH assay detailed in this report overcomes this difficulty and provides a more complete strategy because it recognizes both sides of both breakpoints.

Despite the obvious advantage of this Micro-FISH strategy in delineating complex rearrangements in metaphase cells, the application of these probes to the analysis of interphase nuclei would be of additional importance (eg, obviating the need for metaphase cell populations). While the Micro-FISH products do provide a regionally localized signal over interphase nuclei, they are not sufficiently intense to permit scoring of interphase nuclei for the presence of the Ph chromosome. We are currently working to improve the regional specificity (by modifying the size of the dissected segment and other approaches) to generate probes that both span the translocation breakpoint and provide useful interphase signals.

For several important reasons, the application of our Micro-FISH assay as an adjunct to routine cytogenetic analysis in CML may be of considerable utility. First, there is an extremely high success rate in identifying labeled sites using our high-sequence complexity microdissection probes (approaching 100% of metaphase cells in all experiments to date). Second, the localization of a Ph chromosome by this assay is virtually independent of the quality of the metaphase cell examined. Thus, this approach provides unequivocal results in the majority of cases. Slides for some patients in this study were taken from stored diagnostic pellets that had remained in fixative for almost 5 years before our analysis. Third, the Ph Micro-FISH assay is very straightforward in its interpretation. For example, normal cells always have four labeled sites (two red/orange and two green) (Fig 2B). In contrast, rearranged chromosomes juxtapose red/orange with green fluorescence that is identified as yellow, a color never seen in normal cells (Figs 2C and 3). Also, because of the distinctive red/orange fluorescence of the 22q11 probe, it is particularly easy to track its involvement. Thus, in normal cells there will be two red/orange signals, while in all cases of Ph+ cells, there will only be one red/orange signal. Finally, for all complex translocations, five or more labeled sites (in contrast to four sites in normal cells) will be observed. For example, a three-way translocation will result in the recognition of two red/orange, two green (one with a quantitatively reduced signal), and one yellow labeled site (Fig 3).

While this study has focused on Ph+ CML, this strategy can, of course, also be extended to translocation breakpoints in other malignancies. With the limitation that the currently available probes are principally useful for metaphase cells, this microdissection-based strategy provides a new and valuable tool for the analysis of rearranged chromosomes in human malignancy in general and in CML specifically.

**REFERENCES**

clustered within a limited region, bcr, on chromosome 22. Cell 36:93, 1984


Application of chromosome microdissection probes for elucidation of BCR-ABL fusion and variant Philadelphia chromosome translocations in chronic myelogenous leukemia

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