Highly Sensitive Fluorescence In Situ Hybridization Method to Detect Double BCR/ABL Fusion and Monitor Response to Therapy in Chronic Myeloid Leukemia


We investigated a new method using fluorescence in situ hybridization and DNA probes that span the common breakpoints of t(9;22)(q34;q11.2) and that detect double BCR/ABL fusion (D-FISH) in bone marrow cells with this translocation, one on the abnormal chromosome 9 and one on the Philadelphia chromosome (Ph chromosome). D-FISH patterns were abnormal in 30 of 30 specimens with classic, simple, complex, and masked Ph chromosomes. Based on 200 nuclei from each of 30 normal specimens, the mean percentage of false-positive cells was 0.25 ± 0.39. Thirty-seven specimens from 10 patients were studied before treatment and two or more times at 4-month intervals after treatment with interferon-α2b (IFN-α2b) with or without ara-C. Based on 200 nuclei, the results of D-FISH in these specimens correlated closely with quantitative cytogenetics and accurately quantified disease within a few percent. We studied 6,000 nuclei for each of six specimens, three normal and three from patients with chronic myeloid leukemia (CML) in cytogenetic remission. The normal cutoff for 6,000 nuclei was 0.079% and patients in cytogenetic remission had residual disease ranging from 7 (0.117%) to 53 (0.883%) Ph-positive nuclei. We conclude that D-FISH can detect the Ph chromosome and its variant translocations and accurately quantify disease in CML at diagnosis and at all times after treatment, including cytogenetic remission.

MULTIPLE GENETIC TESTING methods are used in clinical practice to assess response to therapy in chronic myeloid leukemia (CML), but no one technique accurately detects and quantifies disease at diagnosis and at all times during treatment.1-6 A new method, using fluorescence in situ hybridization (FISH), now appears to meet this need in clinical practice. This new method uses FISH and commercially available differently colored BCR and ABL probes that span the common breakpoints of t(9;22)(q34;q11.2) and show double BCR/ABL fusion (D-FISH) in cells with this translocation, one on the abnormal chromosome 9 and one on the Ph chromosome. With D-FISH, the number of false-positive and false-negative cells approaches zero.

Conventional FISH methods with differently colored BCR and ABL DNA probes detect a single BCR/ABL fusion signal on the Ph chromosome (S-FISH).7 S-FISH is highly accurate for analysis of metaphases but is imprecise for the study of interphase cells because BCR and ABL signals coincidentally overlap in about 4% of normal nuclei (false-positive cells).8 Moreover, BCR/ABL fusion signals can be incorrectly scored in many Ph-positive nuclei (false-negative cells) because scoring fusion signals with S-FISH is subjective.9 These technical artifacts limit the potential of S-FISH to detect and quantify minimal residual disease accurately and to measure small fluctuations in the percentage of Ph-positive cells in response to therapy.

Dewald et al10 established that the normal range of S-FISH for interphase bone marrow nucleus is 10% or less, and the abnormal reference range for most untreated patients with CML is 69% to 92%. The statistical method used to determine the normal cutoff was intentionally calculated in a conservative fashion to avoid making false-positive diagnoses of untreated CML. In our experience, this cutoff has worked well in clinical practice to diagnose CML but is high for assessing residual disease in some patients with CML who are receiving therapy. Cox Froncillo et al10 used a more liberal normal cutoff to monitor patients with CML who have been treated, suggesting that detection of 7% or greater of cells with BCR/ABL fusion indicates residual disease.9 Alternative FISH strategies that use either three differently colored probes for BCR, ABL, and ASS (a focus on the centromeric side of ABL on chromosome 9) or differently colored probes that span either the breakpoints in BCR or ABL have been reported to be useful to detect Ph-positive cells in less than 1% of nuclei.10-12 The efficacy of these techniques to detect variant Ph chromosomes and the clinical value of these methods have not been tested.

In a three-part study, we tested D-FISH (1) on various kinds of Ph anomalies and determined its normal range, (2) to monitor patients on treatment for CML, and (3) to detect and quantify low levels of residual disease. The kinds of Ph chromosomes tested included classic t(9;22)(q34;q11.2) anomalies, as well as simple, complex, and masked Ph chromosomes. We also studied specimens with t(9;22)(q34;q11.2) and a breakpoint in the minor BCR region. To test the efficacy of D-FISH for monitoring patients receiving therapy, we studied specimens from 10 patients with CML before and after treatment with interferon-α2b (IFN-α2b) with or without cytosine arabinoside (ara-C). We found the results of D-FISH to correlate well with quantitative cytogenetic methods and were able to determine the percentage of abnormal nuclei accurately within a few percentage points. In testing the limits of resolution to detect Ph-positive nuclei with D-FISH in three normal specimens and in two patients in cytogenetic remission, we detected residual...
RESULTS

Preliminary Studies With Various Kinds of Ph Chromosomes

Preliminary studies were done on metaphases and nuclei from bone marrow of four patients with several types of Ph chromosomes: a t(9;22)(q34;q11.2) with a breakpoint in the major BCR region, a t(9;22)(q34;q11.2) with a breakpoint in the minor BCR region, a t(5;9;22)(q31;q34;q11.2), and a masked Ph chromosome.

In metaphases with a t(9;22)(q34;q11.2) and either a major or minor BCR, we observed a BCR/ABL fusion signal at 22q11.2 on the Ph chromosome, an ABL/BCR fusion signal at 9q34 on the abnormal chromosome 9, a BCR signal at 22q11.2 on the normal chromosome 22, and an ABL signal at 9q34 on the normal chromosome 9 (Fig 1C). In interphase, the D-FISH patterns were mostly 1R1G2F or 2R2G1F. Nuclei with 2R2G1F resulted from separation of the BCR and ABL probes on the abnormal chromosome 9. We noted a predominance of nuclei with 1R1G2F in t(9;22)(q34;q11.2) with a minor BCR breakpoint. In t(9;22)(q34;q11.2) with a major BCR breakpoint, nuclei with 2R2G1F were prevalent.

In metaphases with a t(5;9;22)(q31;q34;q11.2), we observed a BCR/ABL fusion signal at 22q11.2 on the Ph chromosome, a small ABL signal on the abnormal chromosome 9, a small BCR signal on the abnormal chromosome 5, a BCR signal at 22q11.2 on the normal chromosome 22, and an ABL signal at 9q34 on the normal chromosome 9 (Fig 1F). In interphase, the predominant D-FISH pattern was 2R2G1F.

In metaphases with a masked Ph chromosome, we observed a BCR/ABL fusion signal at 22q11.2 on the abnormal chromosome 22, a small ABL signal on the abnormal chromosome 9, a BCR signal at 22q11.2 on the normal chromosome 22, and an ABL signal at 9q34 on the normal chromosome 9 (Fig 11). In interphase, the predominant D-FISH pattern was 1R2G1F. This signal pattern is consistent with a masked Ph chromosome.
derived by insertion of a portion of ABL into BCR and represents an atypical D-FISH pattern.

To determine the initial scoring criteria, we recorded all the signal patterns in 100 consecutive nuclei for each of the preliminary specimens and recognized 34 different signal patterns. These patterns included the five we used in the scoring criteria and two that we expected to encounter for masked Ph chromosomes. The remaining 27 patterns represented various types of technical problems, such as signal overlaps, signal separation, and lack of probe hybridization.

**Typical D-FISH Patterns**

In part I of the investigation, we tested the scoring criteria for 65 bone marrow specimens, including 35 that were normal and 30 with various kinds of Ph chromosomes (Table 1). We found the nuclei in 57 of 65 specimens met the scoring criteria (Fig 1A, B, D, and E), but eight had nuclei with atypical but abnormal D-FISH patterns.

**Normal specimens.** We studied 35 normal specimens, and each one met the scoring criteria. Among the 7,000 nuclei from these specimens, 6,985 (99.79%) were 2R2G. Each of the 15 nuclei with abnormal D-FISH patterns involved split BCR and ABL signals, suggesting that these cells were in G2 of the cell cycle (Fig 1K, false-positive).

**t(9;22) only.** We studied five specimens with only a t(9;22) (Table 1). Each of these specimens met the scoring criteria. Among the collective 1,000 nuclei from these specimens, the D-FISH patterns were 2R2G (2.6%), 1R1G2F (72.8%), and 2R2G1F (24.6%). Thus, 97.4% of nuclei from these five untreated patients were Ph positive and 2.6% were normal.
We studied five specimens with a t(9;22) and either an extra Ph chromosome or trisomy 8 (Table 1). Three of these specimens (nos. 6, 8, and 10) met the scoring criteria. Among the 600 nuclei from these specimens, the D-FISH patterns were 2R2G (4.5%), 1R1G2F (72.3%), 2R2G1F (20.5%), and either 1R1G3F or 2R2G2F (2.7%). Thus, 95.5% of nuclei from these three specimens were Ph positive and 4.5% were normal.

Complex Ph chromosomes. We studied five specimens with complex Ph chromosomes (Table 1). Four of these specimens (nos. 11, 13, 14, and 15) met the scoring criteria. Among the 800 nuclei from these specimens, the D-FISH patterns were 2R2G (2.1%), 1R1G2F (8.9%), and 2R2G1F (89.0%). Thus, 97.9% of nuclei from these four specimens were Ph positive, and 2.1% were normal.

Simple Ph chromosomes. We studied five specimens with simple Ph chromosomes (Table 1). Each of these specimens met the scoring criteria. Specimen 19 was mosaic as 2 of 20 metaphases had a Ph chromosome, and 12.0% of nuclei were Ph positive by D-FISH analysis. Among the 800 nuclei from the nonmosaic specimens, the D-FISH patterns were 2R2G (6.9%), 1R1G2F (11.1%), and 2R2G1F (82.0%). Thus, 93.1% of nuclei were Ph positive, and 6.9% of nuclei were normal in these four nonmosaic specimens. The D-FISH patterns in these specimens
were similar to four of the specimens with a complex translocation.

Ph chromosomes in ALL. We studied five specimens from patients with Ph-positive ALL (Table 1). Each of these specimens met the scoring criteria, regardless of whether the BCR breakpoint was in the major (nos. 26 and 28) or minor (nos. 27, 29, and 30) region. Four of these specimens (nos. 27 through 30) were mosaic for normal and Ph-positive metaphases by cytogenetics. Specimen 26 was nonmosaic, and the D-FISH patterns were 2R2G (0.5%), 1R1G2F (70.0%), and 2R2G1F (29.5%). Thus, 99.5% of nuclei from this specimen were Ph positive and 0.5% were normal.

Atypical D-FISH Patterns

In part I of the investigation, eight specimens had nuclei with an abnormal D-FISH pattern (Table 1), but the signal pattern was different from those used in the scoring criteria. Three of the specimens (nos. 9, 7, and 12) with an atypical D-FISH pattern were in the category of t(9;22) plus other anomalies and one was classified with the complex Ph chromosomes. The five other specimens with atypical D-FISH patterns all had masked Ph chromosomes (nos. 21 through 25).

Complex Ph chromosomes. Specimen 12 had a t(9;22; 19)(q34;q11.2;q13.3); D-FISH patterns were 2R2G (6.0%) and 1R2G1F (94.0%). Metaphases from this specimen had BCR/ABL fusion on the Ph chromosome, but no ABL or BCR signal on the abnormal chromosome 9 (Fig 1L). In specimen 7, D-FISH patterns were 2R2G (5%), 1R1G1F (27%), 1R1G2F (55%), and 2R2G1F (14%). In each Ph-positive metaphase, no ABL or BCR signal was observed on the abnormal chromosome 9. Metaphases from this specimen demonstrated an atypical D-FISH pattern. Some metaphases had one Ph chromosome, but most had two. Thus, nuclei with 1R1G2F or 2R2G1F signals most likely represent cells with two Ph chromosomes.

Masked Ph chromosomes. We studied five specimens with a masked Ph chromosome and found each to have an atypical D-FISH pattern. Specimens 21 and 24 had 94.5% and 78.0% nuclei with 1R1G1F signals, respectively (Fig 1G). Metaphases from each of these specimens had BCR/ABL fusion on the Ph chromosome, but no BCR or ABL signal on the abnormal chromosome 9.

Specimens 23 and 25 had 10.5% and 74.0% nuclei with 1R2G1F signals, respectively (Fig 1J). Metaphases from each of these specimens had a BCR/ABL fusion on the Ph chromosome and a small residual ABL signal on the abnormal chromosome 9 (Fig 1J). This finding is consistent with an insertion of a portion of ABL into the BCR locus.

Specimen 22 had 93.0% nuclei with 2R1G1F signals (Fig 1H). Metaphases from this specimen had an ABL/BCR fusion on the abnormal chromosome 9 and a small residual BCR signal on the abnormal chromosome 22. This is consistent with an insertion of a portion of BCR into the ABL locus.

Normal Range

Based on 200 nuclei from each of 30 normal specimens, the mean percentage and standard deviation of nuclei with scorable D-FISH patterns other than 2R2G was 0.25 ± 0.39, within a range of 0 to 1.5. We calculated the upper bound of a one-sided 95% confidence interval for observing 3 of 200 (1.5%) aberrant cells using the binomial distribution. This implied a cutoff of greater than 7 (3.83%) nuclei with scorable D-FISH patterns other than 2R2G in 200 nuclei to classify any specimen as abnormal.

We classified each of the 35 other specimens in part I of the investigation as normal or abnormal based on the 3.83% normal cutoff. Each of the five normal specimens used to test the cutoff was classified correctly. Among the 30 specimens with a Ph chromosome, 29 were classified as abnormal. The results of specimen 27 were within normal limits based on finding 3.5% abnormal nuclei. This specimen had 2 of 20 (10%) metaphases with a Ph chromosome by conventional cytogenetics.

Abnormal Reference Range

We used the observed values from the D-FISH findings in the 16 patients (nos. 1-6, 8, 10, 13-18, 20, 26) with only a t(9;22) or a variant Ph chromosome in all metaphases, to establish an abnormal reference range. Among these specimens, the percentage of abnormal nuclei ranged from 90.0 to 99.5, with a mean of 96.1.

We tested the precision of D-FISH to identify abnormal nuclei in cells collected by apheresis from a patient with CML in blast crisis and a 9;22 translocation. We processed and analyzed samples from this specimen on 10 occasions and found the percentage of abnormal nuclei ranged from 97 to 100, with a mean of about 98.2.

Unscorable Nuclei

Among the 35 normal specimens from part I of the investigation, the mean number of unscorable cells encountered to acquire 200 scorable nuclei was 152 ± 21.9, within a range of 70 to 240. This number was comparable for each of the microscopists. Among the 30 abnormal specimens from part I, the mean number of unscorable cells encountered to acquire 200 scorable nuclei was 287 ± 77.7, within a range of 98 to 698.

Monitoring Response to Therapy for CML

In part II of the investigation, we used Q-cytogenetics, S-FISH, and D-FISH to study 37 bone marrow specimens from 10 patients (Fig 2). For each patient, we studied one specimen collected before treatment and two or more specimens at approximately 4-month intervals after treatment with IFN-α2b with or without ara-C, according to protocol. These 10 patients included seven with a classic t(9;22)(q34;q11.2) and a typical D-FISH pattern.

Three patients in part II of the investigation had an atypical D-FISH pattern. Patient 2 had nuclei with 1R2G1F resulting from BCR/ABL fusion on the Ph chromosome and a small residual ABL signal, but no BCR signal, on abnormal chromo-
some 9 (Fig 1J). Patient 7 had nuclei with 1R1G1F resulting from BCR/ABL fusion on the Ph chromosome, but no BCR or ABL signal on the abnormal chromosome 9 (Fig 1G). Patient 6 had a masked Ph chromosome, and the D-FISH pattern was consistent with an insertion of a portion of ABL into the BCR locus (Fig 1J).

This series included samples of five different artificial mosaics created by mixing cells from normal specimens with cells from a specimen with a classic t(9;22)(q34;q11.2). The percentage of abnormal nuclei in each of these samples as determined by D-FISH was closer to the expected proportion of Ph-positive nuclei than S-FISH (Fig 1J).

Based on cytogenetic studies during the course of therapy, six patients (nos. 2, 4, 5, 7, 8, and 9) attained less than 33% Ph-positive cells and 3 (nos. 1, 3, and 10) showed no notable change in percentage of Ph-positive metaphases (Fig 2). In assessing the results in seven specimens with typical D-FISH patterns, we found the results of D-FISH to be a better predictor of the findings by Q-cytogenetics than S-FISH based on a mixed model regression analysis, adjusting for within subject correlations.

Patients 2 and 7 had atypical D-FISH patterns and eventually attained more than 67% normal metaphases in response to therapy. In each of these specimens, the percentage of abnormal nuclei by D-FISH was somewhat comparable to S-FISH but still correlated strongly with changing proportions of abnormal nuclei after therapy. Patient 6 had a masked Ph chromosome that was not detected by conventional cytogenetics but that was detected by both D-FISH and S-FISH methods. The percentage of abnormal nuclei for this patient never fell below 80% based on D-FISH. The results of patients 2, 6, and 7 show that D-FISH can be used to monitor response to therapy, even when the Ph anomaly is associated with an atypical signal pattern.

**Resolution Limits to Detect Residual Disease**

In part III of the investigation, we tested the hypothesis that analysis of more than 200 nuclei with D-FISH would significantly increase the ability to detect low levels of Ph-positive nuclei. To test this possibility, 6,000 nuclei from each of six specimens were studied in a blind fashion. Three were from normal specimens from part I of the investigation and three specimens were from two patients (nos. 5 and 8) with CML in cytogenetic remission from part II.

We observed one aberrant cell among 6,000 nuclei in only one of the three specimens from normal individuals (Table 2). On the basis of this finding, we calculated the upper bound of a one-sided 95% confidence interval for observing 1 of 6,000 (0.0167%) aberrant cells using the binomial distribution. This implied a normal cutoff of greater than 0.079% (>4) aberrant cells in 6,000 nuclei to classify any specimen as abnormal.

We compared this cutoff with the results for each of the three specimens from patients with CML in cytogenetic remission (Table 2). One specimen, from patient 8, was within normal D-FISH limits for 200 nuclei after 261 days of treatment. In 6,000 cells, we found 53 (0.883%) abnormal nuclei. Patient 5 had two specimens within normal D-FISH limits for 200 nuclei after 272 days and 391 days of treatment. In 6,000 nuclei, we found 21 (0.350%) abnormal nuclei in the first of these two specimens and 7 (0.117%) in the other. Thus, in each specimen these results exceeded the normal cutoff and were consistent with residual disease.

**DISCUSSION**

**True D-FISH**

A true D-FISH method would detect a BCR/ABL fusion on the Ph chromosome and an ABL/BCR fusion on the abnormal chromosome 9 in cells with a t(9;22)(q34;q11.2). Thus, normal...
cells would have four signals, 2R2G, and nuclei with a t(9;22)(q34;q11.2) would have six signals, 1R1G2F, with fusion signals counting as two hybridization sites.

With the current D-FISH strategy, the ABL/BCR fusion on chromosome 9 was observed as separate ABL and BCR signals in as many as 24% of nuclei with a 9;22 translocation. We scored this pattern as 2R2G1F because we defined a fusion signal as touching or merging BCR and ABL signals. Nevertheless, we classified nuclei with 2R2G1F as Ph positive because they had six BCR and ABL signals; the same as the true D-FISH pattern of 1R1G2F.

With D-FISH, incorrectly scoring a normal nucleus (2R2G) as abnormal (1R1G2F or 2R2G1F) would be rare. Such false-positive observations would require multiple events, including separation of BCR and ABL signals and random overlap of at least one BCR with an ABL signal. Our results are consistent with this conclusion, as we observed only one false-positive cell in 18,000 nuclei in part III of this investigation and 15 of 7,000 nuclei in part I.

The accuracy of scoring with D-FISH improved with experience. In part I of the investigation, we found 15 false-positive nuclei among 7,000 cells from 35 normal individuals. In part III, we found only 1 false-positive nucleus in 18,000 cells from three normal specimens. Part I was performed first, when our technologists had the least experience with scoring D-FISH signals. Part III was performed last and benefited from the learning experience of scoring cells in parts I and II.

Incorrectly scoring abnormal nuclei (1R1G2F or 2R2G1F) as normal (2R2G) would also be rare. Observation of a false-negative nuclei would require multiple events, including the separation of all BCR/ABL fusion signals in a nucleus and random overlap of two BCR signals with each other and two ABL signals with each other. Scoring true-positive cells is reproducible with D-FISH, as the percentage of Ph-positive nuclei that we found in the same specimen processed on 10 separate occasions ranged from 97 to 100, with a mean of 98.2.

D-FISH is a good method for quantifying disease even in untreated patients with CML. The frequency of normal cells in five untreated patients from part I of the investigation with only a 9;22 translocation ranged from 95.0 to 98.5, with a mean of about 97.4. We believe that the few nuclei with a normal pattern represent true normal cells, rather than false-negative observations, because of the objectivity of scoring D-FISH, and because not all cells in each hematopoietic compartment are usually involved in the neoplastic process in CML.

We intentionally used strict scoring criteria in this investiga-

### Table 2. Limits of D-FISH Resolution for Detection of Ph-Positive Cells in 6,000 Nuclei

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Diagnosis</th>
<th>Treatment (days)</th>
<th>Abnormal Nuclei* (%)</th>
<th>Analysis Time (h:min)</th>
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<tr>
<td>Part I, no. 44</td>
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<td>0</td>
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<td>0</td>
<td>0.000</td>
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<tr>
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<td>272</td>
<td>21</td>
<td>0.350</td>
</tr>
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<td>Cytogenetic remission</td>
<td>391</td>
<td>7</td>
<td>0.117</td>
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<tr>
<td>Part II, pt.8, no. 3</td>
<td>Cytogenetic remission</td>
<td>261</td>
<td>53</td>
<td>0.883</td>
</tr>
</tbody>
</table>

*Normal cutoff is >4 abnormal nuclei or more precisely ≥0.079%.

**D-FISH Patterns in Various Kinds of Ph Chromosomes**

In 57 of 65 specimens in part I of the investigation, the D-FISH patterns met the scoring criteria. Except for masked Ph chromosomes, typical D-FISH patterns were found in specimens from each of the different kinds of Ph chromosomes (Table 1). With D-FISH, complex Ph chromosomes can be distinguished from classic 9;22 translocations by virtue of a higher proportion of abnormal nuclei with 2R2G1F. In complex translocations, a BCR/ABL fusion occurs on the Ph chromosome, and no reciprocal ABL/BCR fusion occurs because the telomeric portion of BCR is translocated to another chromosome. We saw similar proportions of nuclei with 2R2G1F in specimens with simple and complex Ph chromosomes. This is consistent with the findings and conclusions of others who suggest that simple Ph chromosomes are actually subtle, complex Ph chromosomes.7,14

Thus, excluding the five specimens with a masked Ph chromosome, in part I of the investigation, 22 of 25 specimens with a Ph chromosome met the scoring criteria. In part II, we studied 10 patients selected because we had three or more specimens to test the efficacy of D-FISH to monitor therapy. Excluding the single patient with a masked Ph chromosome, seven of nine patients met the scoring criteria. These findings may suggest that approximately 78% to 88% of patients with CML in clinical practice would have a D-FISH pattern that would meet these scoring criteria.

**Atypical D-FISH Patterns**

In this study, eight specimens from part I and three patients from part II had abnormal but atypical D-FISH patterns. The scoring criteria were not designed for masked Ph chromosomes. Nevertheless, we expected to observe nuclei with either 1R2G1F or 2R1G1F in these specimens, because masked Ph chromosomes result from the submicroscopic insertion translocation of either a portion of ABL into BCR or vice versa.7 The masked Ph chromosome in specimens 23 and 25 of part I, and patient 6 in part II, had a portion of ABL translocated into the BCR locus.

Specimen 22 from part I, had a D-FISH pattern that was consistent with an insertion of a portion of BCR into the ABL locus.

Two other specimens from part I of the investigation (nos. 21 and 24) had a masked Ph chromosome with no BCR or ABL signal on a chromosome 9, but they did have a BCR/ABL fusion on a chromosome 22. This could result by an insertion translocation of ABL into BCR, but the two breakpoints to excise ABL would have had to occur outside the hybridization sites. Moreover, specimens 7 and 9 from Part I, and patient 7 from part II, had typical 9;22 translocations, but lacked a BCR and ABL signal on the abnormal chromosome 9. This could
result from a break on chromosome 9 on the centromeric side of the ABL hybridization site and a break on chromosome 22 on the telomeric side of BCR.

The aforementioned theories for the origin of atypical D-FISH patterns in masked and 9;22 translocations are problematic, as each of these mechanisms should produce a BCR/ABL fusion signal on the Ph chromosome approximately twice the usual size. Subjectively, the BCR/ABL fusion signal in these specimens did not appear larger than usual, favoring an alternative theory. It is possible that the breakpoints are within the usual BCR and ABL locus, but the ABL/BCR hybridization site for chromosome 9 may have been lost by a microdeletion process during the formation of some masked Ph chromosomes and 9;22 translocations.

Patient 2 in part II of the investigation had a typical 9;22 translocation, but the D-FISH pattern was 1R2G1F. Metaphases had no BCR signal on chromosome 9, a small residual ABL signal was present on the abnormal chromosome 9. This could result from either a microdeletion of one of the BCR hybridization site or a break on the Ph chromosome that was on the telomeric side of the BCR hybridization site.

Specimen 12 from part I of the investigation had a t(9;22;19) anomaly and an atypical D-FISH pattern, 1R2G1F. Metaphases had a BCR/ABL fusion on the Ph chromosome, a small residual ABL on the abnormal chromosome 9, but no BCR signal on the abnormal chromosome 19. This could result from a break in the usual ABL locus and a break on the telomeric side of BCR or a microdeletion process that resulted in loss of a portion of the BCR locus.

Although the scoring criteria did not include these atypical patterns, these specimens were clearly abnormal and required special scoring criteria. For investigators who will use D-FISH, it will be important to account for these atypical patterns on a patient-to-patient basis. For example, it appears from patients 2, 6, and 7 in part II that the atypical patterns persist in cells before treatment and throughout the treatment period. We did not attempt to calculate normal or abnormal reference ranges for specimens with atypical patterns, because they varied and the sample size was small. We expect the percentage of false-positive and false-negative nuclei to be higher in these atypical cases than for patients who meet the D-FISH scoring criteria.

Monitoring Response to Treatment

Based on analysis of 200 nuclei from each of the 30 normal specimens in part I of the investigation, we calculated an upper limit of the normal range at 3.83% abnormal nuclei. This is a conservative estimate of the normal cutoff, because it is based on using the maximum number of false-positive cells in any one patient in a series (eg, 1.5%) and then calculating the 95th percentile for all normals using the binomial distribution. We used a similar statistical method to calculate a normal cutoff for S-FISH at 10%, a figure that has served us and others well in clinical practice to arrive at an accurate diagnosis of CML.

The percentage of Ph-positive metaphases as determined by conventional cytogenetic methods is considered the current "gold standard" for measuring response to therapy in CML. Most cytogenetic laboratories analyze 20 metaphases selected primarily on the basis of metaphase morphology. This approach is adequate to detect cells with a Ph chromosome but is susceptible to sample error and bias toward metaphases with good morphology. These factors can interfere with the reliability of conventional cytogenetics to quantify residual disease in response to therapy. Consequently, we have modified the conventional method to select consecutive metaphases in which the 9q34 and 22q11.2 regions of chromosomes 9 and 22 are visible, placing less emphasis on quality of metaphase morphology. We refer to this method as quantitative cytogenetics or Q-cytogenetics. In comparing the results of Q-cytogenetics–based analysis of 25 metaphases with the results of analysis of 200 consecutive metaphases using S-FISH, we have found that the results are usually within approximately 5%. Thus, we believe Q-cytogenetics is an efficacious and cost-effective method to quantify proliferating disease in CML.

Each of the specimens from patients in part II of the investigation was studied using Q-cytogenetics, S-FISH, and D-FISH. For the seven patients who had “true D-FISH” patterns, the results of D-FISH were a significantly better predictor of the results of Q-cytogenetics than S-FISH. Based on analysis of only 200 nuclei, D-FISH is comparable to cytogenetics before and after treatment. However, D-FISH would be far superior to S-FISH to monitor patients with CML on therapy (Fig 2).

We did not attempt to correlate the results of D-FISH with any clinical parameter or survival. Because the results correlate well with cytogenetics, D-FISH should prove to have comparable prognostic value, but this will not be known until D-FISH is used to study a large series of patients over a long period of time.

Limits of Resolution to Detect Residual Disease

Based on analysis of 200 nuclei with D-FISH, in part I of the investigation we found that it is possible to detect residual disease corresponding to 3.83% or greater abnormal cells. In part III, we found that, with D-FISH, the limits of detecting residual disease could be reduced to 0.079% by analysis of 6,000 nuclei. For patients in part II of the investigation, only three specimens fell within normal limits for D-FISH, based on analysis of 200 nuclei. Each of these specimens had only normal metaphases by Q-cytogenetics. However, based on analysis of 6,000 nuclei, using D-FISH, each specimen had residual disease ranging from 7 (0.117%) nuclei in one specimen to 53 (0.883%) nuclei in another.

The effort needed to score 6,000 nuclei ranged from approximately 2.5 to 3.25 hours, compared with 5 to 10 minutes to score 200 nuclei. Perhaps a cost-effective strategy to use D-FISH for monitoring patients with CML receiving therapy would be to study 200 nuclei in bone marrow specimens collected before treatment to establish a baseline for the percentage of Ph-positive cells and to determine whether the D-FISH pattern is typical or atypical. If the patient has a typical D-FISH pattern for a Ph chromosome, D-FISH can be used after treatment until the patient achieves a level of less than 3.83% abnormal nuclei. The normal cutoff for 200 nuclei approximates cytogenetic remission, ie, 25 of 25 normal metaphases by Q-cytogenetics. For specimens within normal limits for 200 nuclei, it may be useful to study up to 6,000 nuclei to detect residual disease as low as 0.079% Ph-positive cells. If the
patient has an atypical D-FISH pattern, a different normal cutoff would have to be established.

In conclusion, D-FISH is a significant technological advancement in monitoring therapy for CML. D-FISH detects all variant translocations of the Ph chromosome and accurately quantifies disease in CML at diagnosis and at all times during treatment, even for patients in cytogenetic remission.

REFERENCES


4. Lion T, on behalf of the European Investigators on Chronic Myeloid Leukemia Group: Clinical implications of qualitative and quantitative polymerase chain reaction analysis in the monitoring of patients with chronic myelogenous leukemia. Bone Marrow Transplant 14:505, 1994


Highly Sensitive Fluorescence In Situ Hybridization Method to Detect Double BCR/ABL Fusion and Monitor Response to Therapy in Chronic Myeloid Leukemia