Quantification of the Breakpoint Cluster Region Rearrangement for Clinical Monitoring in Philadelphia Chromosome-Positive Chronic Myeloid Leukemia

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The purpose of this report was to evaluate scintigraphy analysis of Southern blot hybridization as a method to quantify the breakpoint cluster region (BCR) rearrangement of Philadelphia chromosome (Ph+) chronic myelogenous leukemia (CML). Cytogenetic and molecular studies performed simultaneously on 47 bone marrow and/or blood samples from 300 patients treated with α-interferon-based therapy were compared. Molecular results were expressed as the percentage of rearranged BCR bands versus the total scintigraphic signal. The percentage of Ph+ metaphases was calculated on 25 metaphases. The results of molecular studies obtained on both peripheral blood and bone marrow samples were identical. The rank correlation between the BCR quantification and the percentage of Ph positivity in 465 samples was excellent (r = .78). However, of 99 samples with a normal karyotype, 24% had a BCR rearrangement. Of 86 samples with no BCR rearrangement, 13% showed a Ph chromosome.

Of 49 samples with partial cytogenetic remission (Ph+ metaphases, 1% to 34%), 23% had no BCR rearrangement. In samples with a minor or no cytogenetic response (Ph+ metaphases, >34%), BCR analysis underestimated the degree of response in 73 of 326 samples (22%). Nevertheless, survival analysis by BCR quantification showed statistically better outcome for patients in complete or partial molecular response (P < .01). Molecular quantification of BCR was useful in monitoring the course of Ph+ CML. This method, which can be used on peripheral blood, detected residual disease not shown by cytogenetic analysis and was prognostically relevant as a measure of disease suppression.

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PHILADELPHIA chromosome (Ph+) chronic myelogenous leukemia (CML) is a disease of the hematopoietic stem cell that progresses in a multistep fashion. The reciprocal translocation between chromosomes 9 and 22 is the hallmark of CML. The breakpoint on chromosome 22 occurs most often within a 5.8-kb region located between exons 1 and 4 of the breakpoint cluster region (BCR) gene, which is called the major breakpoint cluster region. In patients on therapy, this translocation has been monitored by karyotype analysis of bone marrow samples. However, this method is limited by (1) a requirement for mitotic cells, (2) the presence of translocations below the level of cytogenetic detection, (3) an insufficient amount of metaphase material or a restricted number of metaphases analyzed in some samples, and (4) its possible low sensitivity to detect the near disappearance of the leukemic clone under therapy.

Detection of the BCR rearrangement by Southern blot analysis has been used as a clinical tool in patients with CML and in patients with acute lymphoblastic leukemia (ALL). To date, Southern blot analysis has been useful in diagnosing patients with Ph+ CML and BCR rearrangement and patients with ALL and p210 Ph+ disease not identified by cytogenetic studies. The intensity of the rearranged bands shown by Southern blotting can be quantified by densitometric analysis of autoradiograph exposure. In this study, we compared BCR rearrangement quantification with routine cytogenetic studies performed in a serial evaluation of patients with Ph+ CML on therapy. The objective of our analysis was to assess whether BCR quantification could be used as an alternative, or as a superior tool, to monitor the efficacy of therapy in patients with CML.

MATERIALS AND METHODS

Patients. Three hundred patients with Ph+ CML treated with α-interferon (IFN-α)-based therapy were included in the study after informed consent was obtained according to institutional guidelines. Samples of blood and/or bone marrow were submitted for molecular analysis of BCR quantification. A bone marrow sample was submitted simultaneously for cytogenetic studies.

Cytogenetic analysis. Routine cytogenetic studies were performed on bone marrow aspirates. Samples were incubated overnight at 37°C in Ham's F-10 medium supplemented with 10% fetal calf serum and Pen-Strep antibiotics. Cultures were harvested and slides were prepared according to established procedures; 25 metaphases were analyzed when available.

Molecular analysis. DNA was purified with standard phenol/chloroform extractions and ethanol precipitation after proteinase K digestion. Ten micrograms of DNA was digested to completion with one of the following restriction endonucleases: EcoRI, BamHI, Bgl II, or Xba I. The digests underwent electrophoresis on a Probe Tech (Gibco) 5% agarose gel before being transferred to a nylon filter, in accordance with the manufacturer's instructions. A solution composed of HindIII and BamHI digests of control DNA in equal proportions and diluted to approximately 5% of normally loaded sample DNA was loaded onto each gel as a quality control. Hybridization studies were performed with either of two oligolabeled DNA probes: the universal bcr probe (Ph-bcr/3), which is a 4.5-kb DNA fragment that contains sequences from the entire bcr region except for a central site; and a 3' probe (bcr[PR-1]), which is a 1.2-kb Bgl II-HindIII fragment that contains only sequences 3' to the central BamHI site.

Quantitative molecular analysis. After hybridization, Southern blots were scanned for 5 to 24 hours with a Betagen scanner (Betta-gen, Waltham, MA) that detects particle emissions and plots the origin of the particle to a location on the filter. Molecular weight

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and counts per minute of the rearranged and germline bands were determined according to the manufacturer's instructions. Scanned filters were then exposed to Kodak X-OMAT AR x-ray film (Eastman Kodak, Rochester, NY) for 3 to 14 days at -70°C to obtain a film record of the rearrangement and to rule out the presence of faint rearranged bands below the limits of resolution of the Betagen scanner. If sensitivity bands were not visible at the shorter exposure period, the filter was exposed to the film for the longer period of time.

Cytogenetic:molecular ratio. In cytogenetic studies, the presence of one Ph+ chromosome is interpreted as a positive event (binary system). In molecular studies, the intensity of the rearranged BCR band is calculated as the ratio of the signal from the rearranged BCR band(s) to the added signals from the rearranged BCR band and the normal unarranged gene. A theoretical 2:1 ratio exists between the cytogenetic and the molecular results. For example, a cell population that is 100% Ph+ would be expected to have a BCR rearrangement value of 50% [the abnormal t(9;22) chromosome/the same + the other normal chromosome]; a 50% Ph+ cell population would have an expected BCR rearrangement of 25% (one quarter of the DNA carries the 9;22 translocation).

Initial studies sought to optimize the ratio of the intensity of the rearranged band versus the total DNA by analyzing probe and enzyme combinations that yielded a single rearranged band as well as a single germline band. The reason for this is that more than one germline band occurs when the probe contains sequences recognizing more than one different portion of the same DNA molecule that have been separated by enzyme digestion and electrophoresis. Therefore, adding the intensity of the bands artificially doubles the contribution of a single DNA molecule to the overall intensity. The same is true in a majority of the cases of more than one rearranged band, in which the intensities of the bands are comparable (the intensity of the rearranged bands obtained on the same sample were averaged if their difference was no more than 75% for the smallest band). Cases in which the intensity of the rearranged bands was different (more than 75%) were thought to possibly represent clonal variations and the intensities of the bands were added. (Although it is possible to have diminished intensity for small size bands [<2 kb], most of the rearranged bands were larger and the intensity was not thought to be affected by the size of the bands.) In the earlier studies there were often two different enzyme digests present on the filter, both of which were scanned and analyzed. These analyses occasionally yielded slightly different ratios. Results obtained on the same sample with different enzymes did not influence the final range assignment.

Statistical methods. Analysis of the correlation between molecular quantification and percentage of Ph+ metaphases was performed using the nonparametric Spearman’s Rank correlation coefficient.1

![Fig 1. Correlation between BCR quantification and percentage of Ph+ metaphases in 465 samples.](Image)
Results obtained from bone marrow and blood samples were analyzed with the two-tailed paired Student’s t-test. Survival was calculated from the date of the sample analysis by the modified Wilcoxon test. Differences in survival were assessed by the log-rank test. When serial studies were performed, the patient was categorized by the best cytogenetic response; survival was calculated from the date of the sample analysis with the best cytogenetic response.

RESULTS

Patient characteristics. All 300 patients had Ph+ CML. Their median age was 42 years and 40% were female. Twenty-eight patients were studied at diagnosis, and 272 patients were studied during therapy with IFN-α. There were 221 patients analyzed in chronic phase: 89 were within 1 year of diagnosis (early chronic phase) and 132 were diagnosed for more than 1 year (late chronic phase). Seventy-nine patients were investigated in CML transformed phases: 54 were in accelerated phase and 25 in blastic phase.

At the time of blood sampling, 4 patients had a double Ph chromosome in 24% to 100% of the analyzed metaphases. Three patients were in blastic phase and 1 was in accelerated
phase. The presence of a double Ph chromosome was not consistently associated with a second rearranged band.

Comparison of cytogenetic and molecular studies. Four hundred seventy-four samples from 300 patients were analyzed. Table 1 shows the distribution of samples by the percentage of Ph' metaphases and the BCR quantification. Ninety-nine samples were from patients who had achieved a complete cytogenetic response (Ph' metaphases, 0%); 75 samples had no BCR rearrangement; and 24 (24%) had a rearrangement. These rearrangements were quantified for 15 samples and ranged from 2.1% to 38.5% (median, 12.4%). In contrast, 11 (3%) of 375 samples with Ph' metaphases had no BCR rearrangement. In these 11 samples, the percentage of Ph' metaphases ranged from 4% to 24% (median, 5%).

Table 2 shows the distribution of samples when categorized by the percentage of Ph' metaphases and the BCR quantification. The cut-off values were chosen to reflect cytogenetic response categories, as previously described.11 A complete cytogenetic response would correspond to a BCR quantification of 0%. A partial cytogenetic response (1% to 34% Ph' metaphases) would correspond to a BCR quantification of 1% to less than 17.5%. A major cytogenetic response includes both complete and partial cytogenetic responses. A minor cytogenetic response (35% to 95% Ph' metaphases) would correspond to a BCR quantification of 17.5% to less than 47.5%.

Of the 238 samples with no cytogenetic response (>95% Ph' metaphases), 29 (12%) had a BCR value less than 17.5%. However, in samples with a minor cytogenetic response (35% to 95% Ph' metaphases), 44 of 88 (50%) had a BCR quantification less than 17.5%. In contrast, in patients with 1% to 34% Ph' metaphases, only 6 of 49 (12%) had BCR quantification ≥17.5%. A good correlation was found between the percentage of Ph' metaphases and the BCR quantification (Fig 1; r = .78; P < .001).

The BCR quantification level was similar in patients investigated at diagnosis, in those in chronic phase with 100% Ph' cells, and in those in accelerated or blastic phases (Table 3). The lower values seen in patients with less than 100% Ph positivity correlates with the values shown in Fig 1.

Prognosis by measurements of the Ph abnormality. Figs 2 and 3 show the survival of patients according to their cytogenetic response and BCR quantification. Both cytogenetic and molecular studies were significant prognostic determinants of survival.

One important question was which of these two studies was a better prognostic determinant in discordant situations. Because the cytogenetic study is currently the gold standard for prognostic monitoring, we evaluated the survival of patients in specific cytogenetic categories by different BCR quantification levels.

Table 4 shows the survival of all patients by both molecular quantification (below or above 17.5%) and cytogenetic response. The survival of patients who did not respond to therapy (>95% Ph' metaphases) is depicted in Fig 4, according to the molecular quantification. There is a trend for higher incidence of survival when the BCR quantification is less than 17.5% (P = .05).

Significance of BCR quantification at diagnosis. All but 1 of the 28 patients tested at diagnosis had greater than 90% Ph' metaphases. Among them, 5 had a BCR quantification less than 17.5%. Patients were divided into two groups (BCR levels <17.5%; 5 patients; ≥17.5%; 23 patients). At this time of follow-up, only one death was recorded among the patients of the latter group. Therefore, no significant conclusion can be drawn regarding differences in survival by BCR values at diagnosis.

BCR quantification in bone marrow versus blood samples. Samples obtained simultaneously from peripheral blood and bone marrow were compared for 79 cases (Table 5). BCR quantification was performed in 72 of these samples. Figure 5 shows the correlation between results obtained on the bone marrow and the peripheral blood. Molecular studies were negative in both for 35 samples of blood and bone marrow and positive in both for 42 samples. A BCR rearrangement was present in 1 sample of peripheral blood, whereas the corresponding sample of bone marrow had none; the cytogenetic analysis showed 5% Ph' metaphases. In 1 case of a patient with a normal karyotype, the marrow sample showed a questionable BCR rearrangement whereas the peripheral blood sample showed none. The Pearson correlation coefficient for the samples that were quantified was .94.

DISCUSSION

Molecular analysis of the BCR rearrangement is currently used in clinical practice to diagnose Ph' CML and ALL. However, very few studies have analyzed the correlation between the conventional cytogenetic analysis and the newer molecular technique for monitoring the courses of these diseases.5,6

Our results in a large number of patients investigated indicate a good correlation between the BCR quantification and the percentage of Ph' metaphases (Fig 1). However, a significant discordance was found in 50% of samples with a minor cytogenetic response in whom quantification of cells with the BCR rearrangement overestimated the degree of response (ie, BCR rearrangement <17.5%). This difference may be explained by the proliferative advantage of the Ph' clone, which results in a higher detection of Ph' metaphases

**Table 2. Distribution of Samples by Cytogenetic Response Categories**

<table>
<thead>
<tr>
<th>% Ph' Metaphases</th>
<th>% BCR Quantification</th>
<th>No. of Patients</th>
<th>Probability of Survival at 2 yr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>41</td>
<td>98</td>
</tr>
<tr>
<td>1 to &lt;17.5</td>
<td>≥17.5</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>≥17.5</td>
<td>4</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>1 to 34</td>
<td>&lt;17.5</td>
<td>25</td>
<td>95</td>
</tr>
<tr>
<td>≥17.5</td>
<td>4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>35 to 95</td>
<td>&lt;17.5</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>≥17.5</td>
<td>29</td>
<td>75</td>
<td></td>
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<tr>
<td>&gt;95</td>
<td>&lt;17.5</td>
<td>21</td>
<td>85</td>
</tr>
<tr>
<td>≥17.5</td>
<td>151</td>
<td>67</td>
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</tbody>
</table>

Abbreviation: NS, not significant.

**Table 4. Survival From the Date of the Best Cytogenetic Response by BCR Quantification Within Cytogenetic Response Categories**

<table>
<thead>
<tr>
<th>% Ph' Metaphases</th>
<th>% BCR Quantification</th>
<th>No. of Patients</th>
<th>Probability of Survival at 2 yr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>41</td>
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<td>151</td>
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</table>
Fig 4. Survival analysis according to the BCR quantification in patients with Ph+ chromosome CML who did not have a response despite therapy. Survival is calculated from the time of the best cytogenetic response.

Several additional questions are of importance. (1) Can BCR quantification detect residual disease in patients for whom cytogenetic studies show no Ph+ metaphases? (2) Can BCR quantification be used for prognostic purposes? (3) In situations of discordant results between cytogenetic and molecular analyses, is BCR quantification a better prognostic determinant than cytogenetic analysis?

It was interesting that BCR rearrangement studies were positive in 24 of 99 samples (24%) in which Ph+ metaphases were not detected by cytogenetic analysis. This finding suggests that molecular studies can identify residual disease that is undetected by cytogenetic studies. Conversely, 11 of 86
samples (13%) without BCR rearrangement showed Ph+ metaphases. Thus, considering only patients who had a negative result by either test, the concordance rate was 68% (75 of 110), the potential detection rate was 22% (24 of 110) by BCR quantification, and the false-negative rate was 10% (11 of 110) (Table 1).

BCR quantification was found to be predictive of survival outcome in patients with Ph+ CML on therapy. With a median follow-up of 78 weeks from the time of sample measurement, patients with high BCR values had a significantly worse survival than those with low BCR values (Fig 3). Despite the relatively short follow-up in the study and the small number of patients by subgroups, we were able to identify a prognostic trend for differences in the BCR quantification levels in patients with no cytogenetic response (Table 4). A low BCR value (<17.5%) might indicate a more favorable prognosis in patients with no cytogenetic response (Fig 4). Of patients identified in this category, one third had the low BCR value detected before the start of therapy, whereas the remaining patients obtained a molecular response while on therapy.

In summary, cytogenetic studies and BCR quantification have shown relatively good concordance in general for the monitoring of patients with Ph+ CML on therapy. As a single variable, BCR quantification was found to be a good prognostic determinant and might be superior to cytogenetic analysis for determining prognosis in discordant samples. The BCR quantification method may be very a useful alternative to cytogenetic studies in patients who have already achieved a major cytogenetic response and require serial monitoring on therapy. However, BCR quantification, as conducted in this study, may overestimate the degree of response in many patients who have no or minor cytogenetic response. Modification of the procedure (eg, BCR quantification of the myeloid cells) may improve the accuracy of BCR quantification monitoring.

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Quantification of the breakpoint cluster region rearrangement for clinical monitoring in Philadelphia chromosome-positive chronic myeloid leukemia

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