Analysis of Breakpoints Within the bcr Gene and Their Correlation With the Clinical Course of Philadelphia-Positive Chronic Myelogenous Leukemia

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Chronic myelogenous leukemia (CML) is characterized by a reciprocal translocation between chromosomes 9 and 22. The breakpoints on chromosome 22 are clustered within a 5.8-kilobase (kb) DNA fragment known as the breakpoint cluster region (bcr), which encodes part of a functionally active gene. We analyzed the bcr in DNAs from 108 consecutive, unselected Philadelphia chromosome-positive CML patients by Southern blot and determined five restriction enzyme fragments within which breaks occur on chromosome 22. The exact sublocalization was determined in the DNA of 100 patients. It was found to be within the 5.8-kb bcr in 99 patients and outside the bcr in only one. Within the bcr, most of the breakpoints occurred in fragments 1, 2, and 3. Overall, laboratory and clinical features of CML did not correlate with specific breakpoint fragments, but chronic-phase duration was longer in patients with a breakpoint in fragment 2 of the bcr. Large 3' bcr deletions were found in nine patients but did not influence clinical outcome. DNA from one of six patients analyzed both during chronic phase and blast crisis showed an additional aberrant fragment, which suggested that a second abnormal clone developed in blast crisis.

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RESULTS

Sublocalization of the bcr breakpoint. We subdivided the bcr gene into defined restriction endonuclease fragments (Fig 1). Representative examples of Southern blots for breaks within each fragment are displayed in Fig 2. As shown in Fig 2, lane C, breaks within fragment 1 (F1) resulted in rearranged and germline fragments when using probe A and restriction enzymes EcoRI and BglII but only germline fragments when using HindIII and BamHI. For breaks in F2 (Fig 2, lane D), we detected rearranged fragments when using probe A and restriction enzymes EcoRI, BglII, and BamHI but not with HindIII. In a similar fashion, we defined the specific breakpoint fragments for each DNA sample. Rearrangements were confirmed by the demonstration of rearranged fragments with both probes or with two different restriction enzymes.

DNA from 108 consecutive, unselected Ph1-positive CML patients was analyzed by Southern blot. In all 108 DNA samples, the bcr gene was rearranged. The location of the breakpoint within the bcr gene, as defined in Fig 1, was determined in 100 patients. In eight patients, the bcr gene was rearranged, but the exact fragment within which the breakpoint occurred, as defined by Fig 1, could not be determined.

The distribution of the patients on the basis of clinical stage and correlated with bcr breakpoint fragments is presented in Table 1. In the majority of patients, the DNA breakpoints occurred within fragments 1, 2, or 3 of the gene (93%). The breakpoint occurred in fragment 0 in only one patient and in fragment 4 in six patients. Thirty-eight patients were examined at the time of blastic crisis, 24 at myeloid blastic crisis, seven in lymphatic, and seven in undifferentiated. There was no difference in the distribution of those patient samples within the breakpoint fragments when comparing chronic phase to blastic crisis and when comparing different histological types of blastic crisis.

Clinical correlations. The correlation between the site of the bcr breakpoint fragment and the patients’ clinical and hematologic features is summarized in Table 2. To standardize our analysis, we compared only the laboratory and clinical features obtained at the time of diagnosis, when all patients were in the chronic phase of CML. Because M.D. Anderson Hospital is a referral institution, presenting features for some patients were sometimes unknown, and these patients were excluded from our statistical analysis.

There was no difference in the initial presenting laboratory values for hemoglobin, WBC count, or platelet count when comparing patients in groups F1 through F4. Clinical features such as age, sex, and palpable spleen were similar among the groups. There was a trend for longer duration of the chronic phase in patients with breakpoints in F2 compared with all other groups (68 ± 59 months, P = .06) as well as when compared with the individual subsets of patients with breakpoints in F1 (68 ± 43 months, P = .07) or in F3 (68 ± 51 months, P = .04). However, there was no statistical difference in the duration of the chronic phase in patients with breakpoints in F1 and F2 compared with F3 and F4 (59 ± 59 months, P = .4).

Deletions of the bcr. As demonstrated in Figs 2 and 3 (panel B1), chromosomal breaks and subsequent translocations that occur within the DNA sequences used as a probe result in two rearranged fragments: one fragment representing 22 q− sequences and one, 9q+ sequences.14 In the 57 samples in which the breakpoint occurred within F1 and F2 when using probe B and the Bcll and BglII restriction

Fig 1. Restriction enzyme map of human bcr locus adapted partially from Heisterkamp et al. Exons are indicated by vertical black boxes below the map. Restriction enzymes: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Bc, Bcll; X, XbaI; S, SstI. The 5.8-kb bcr is arbitrarily divided into four fragments (F1 to F4). The fragment 5' to bcr was designated F0. The probes used are indicated below the map.

Statistical methods. Differences among subgroups of patients were analyzed by using the chi-square test. Chronic-phase duration curves were plotted by using the method of Kaplan and Meier,19 and differences among curves were analyzed by the log rank test.20
ANALYSIS OF BREAKPOINTS WITHIN THE bcr GENE

Table 1. Distribution of Patients in Different Clinical Stages Based on Breakpoint Fragments Within the bcr Gene

<table>
<thead>
<tr>
<th>CML Clinical Stage</th>
<th>No. of Patients</th>
<th>bcr Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F0</td>
</tr>
<tr>
<td>Chronic and accelerated</td>
<td>62</td>
<td>1</td>
</tr>
<tr>
<td>Blastic Crisis</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Myeloid</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

enzymes, we detected rearranged fragments in all instances. We then reprobed these same blots with probe A and detected a single, rearranged band in 48 instances, thus suggesting that probe A was detecting the 9q+ sequences as expected. In nine of the 57 instances in which we detected no rearranged band with probe A, we assume that the 3' bcr sequences, which should have translocated to the 9q+ chromosome, were deleted (Fig 3). In one additional instance, we detected 5' breakpoint and 3' breakpoint. We assume that the region between these two breakpoints was deleted (case 10, Table 3). There were no unique clinical or laboratory features in this group of patients (Table 3).

Among the 36 DNA samples in which the breaks occurred within fragment 3 when we used probes A and B and BamHI, BglII, HindIII, or EcoRI (Fig 2, lane E), we detected two rearranged fragments in 33 instances, thus suggesting that in these instances we were detecting both 22q- and 9q+ sequences. In those three of 36 instances in which we detected only one rearranged fragment, we assume that 3' bcr sequences were deleted. Because we did not have a separate probe 3' to fragment 3, we could not test this directly.

Progression. To determine whether or not the breakpoint within the bcr gene changes as CML patients progress from chronic phase to blastic crisis, we studied six patients during both chronic phase and acute blastic crisis. There was no change in the breakpoints in five patients, and one patient showed an additional aberrant band only with EcoRI digestion and both probes (Fig 3, lane C). A summary of certain laboratory and clinical features of these patients is given in Table 4.

DISCUSSION

The breaks on chromosome 9, band q34, in Ph'-positive CML patients are spread over 200 kb 5' to the second ("common") abl exon.21,22 Despite this wide variability at the DNA level, we and others have demonstrated that the mRNA product always includes this second ("common") abl exon, which provides the acceptor splice site for 5' bcr sequences.7,23,25 Our data in 100 consecutive unselected patients with Ph'-positive CML confirmed that, in contrast to the breaks on chromosome 9, the breaks on chromosome 22, band q11, are clustered within a restricted 5.8 kb bcr.22,26 Every case had its own specific break within both the abl and bcr genes, thereby resulting in wide heterogeneity at the DNA level. The breakpoints are located within introns.6,25 Within bcr, the breaks are located mainly between exons 2 and 3, between exons 3 and 4, or between 4 and 5. From this analysis it is clear that for the bcr portion of the hybrid bcr/abl mRNA, the splice donor side varies. In >24% of the cases, the breaks on chromosome 22 occur so that the "second" bcr exon provides the splice donor site, in >36%, the "third" bcr exon provides this site. After translation at least two different chimeric proteins are synthetized, which may affect the phenotype and the characteristics of the disease.27

Our study was undertaken to determine whether this molecular heterogeneity correlated with the clinical or laboratory features of CML. There was no correlation with sex; age; splenomegaly; or degree of anemia, leukocytosis, or thrombocytosis. However, there was a trend for longer duration of the chronic phase in patients within group 2 than in groups 1 and 3 in which it was similar. This finding is interesting because groups 1 and 3 are homogeneous groups with respect to the exclusion or inclusion of bcr exon 3 within the chimeric bcr/abl mRNA. These data suggest that bcr exon 3 does not influence the clinical course of Ph'-positive CML. In contrast, the localization of the breakpoint in the intron between exons 2 and 3 may possibly influence the duration of chronic phase. Because the efficiency of splicing is in part dependent on the 5' splice region within introns,28 perhaps translocations within this region diminish the efficiency of splicing and formation of the hybrid mRNA in CML. The observed differences in chronic-phase duration according to the bcr breakpoints are, however, marginal and should be interpreted cautiously and confirmed in larger population studies.

Recent reports have shown that some patients with Ph'-positive acute lymphoblastic leukemia (ALL) have translocations to other not yet defined regions of chromosome 22.17,29,30 This observation suggests that sequences other than the usual 5.8-kb bcr fused to c-abl may influence the

Table 2. Correlation of Clinical and Hematologic Features of Ph'-positive CML Patients With the bcr Breakpoint Fragments

<table>
<thead>
<tr>
<th>bcr Breakpoint Fragment</th>
<th>No. of Patients</th>
<th>Age (Median)</th>
<th>Sex (F/M)</th>
<th>Palpable Spleen</th>
<th>Anemia*</th>
<th>WBC (x 10^9/L) (Median)</th>
<th>Platelet Count (&gt;450 x 10^9/L) (Median)</th>
<th>Chronic Phase Duration (Median in mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>24</td>
<td>39</td>
<td>8/16</td>
<td>12 (17)†</td>
<td>7 (20)</td>
<td>113 (20)</td>
<td>7 (21)</td>
<td>43</td>
</tr>
<tr>
<td>F2</td>
<td>33</td>
<td>43</td>
<td>14/19</td>
<td>20 (24)</td>
<td>8 (21)</td>
<td>153 (23)</td>
<td>14 (20)</td>
<td>68</td>
</tr>
<tr>
<td>F3</td>
<td>36</td>
<td>41</td>
<td>10/26</td>
<td>17 (24)</td>
<td>12 (22)</td>
<td>113 (26)</td>
<td>16 (26)</td>
<td>51</td>
</tr>
<tr>
<td>F4</td>
<td>6</td>
<td>40</td>
<td>2/4</td>
<td>5 (6)</td>
<td>1 (1)</td>
<td>210 (5)</td>
<td>4 (6)</td>
<td>69</td>
</tr>
</tbody>
</table>

*Anemia: males, hemoglobin <12 g/dL; females, hemoglobin <11 g/dL.
†Certain criteria were not known for all patients. Numbers in parentheses indicate the number of patients in whom this parameter was known to us at the time of diagnosis.
histological type of leukemia and the aggressiveness of the disease. In our study, no linkage was found between the histological type of blast crisis and location of the bcr breakpoint in samples from Ph'-positive CML patients. A recent report suggests a strong correlation between 3' bcr breakpoints and blast crisis of CML as well as a chronic phase of short duration. In our study, no statistical difference was found between the chronic-phase duration in patients with bcr breakpoints in F1 and F2 (5' bcr) v F3 and F4 (3' bcr). Most of our patients were given a-interferon rather than chemotherapy, and this difference in therapy might account for the discrepancies between our and the other study findings. Likewise, patients with blast crisis had a distribution of bcr breakpoints similar to that seen in the chronic phase patients.

Several studies have demonstrated that 3' bcr deletions occur in sequences that form part of the 9q + chromosome. Popenoe et al found large deletions in four of 14 patients with Ph'-positive CML and suggested that more proximal deletions occurred in four other patients. In the CML cell line K562, 3' bcr sequences are also deleted. In contrast, de-Klein et al, found only small deletions in chromosomes from patients with Ph'-positive CML. Our analysis suggests that large deletions occur in 10% to 20% of patients and are independent of the site of breaks within the bcr. There were no unique features associated with these Ph'-positive CML samples. An even higher incidence of 3' bcr deletions was found in patients with Ph'-positive ALL. Deletions probably occur at the time of translocation and may be involved in the mechanism of Ph chromosome recombination. Because of deletions and overlapping fragments, the use of a single 3' bcr probe and a single restriction enzyme is not sufficient to detect all Ph'-positive CML bcr rearrangements. We suggest the use of a large 5' bcr probe and at least two restriction enzymes (BglII, BclI).

To determine whether a new clone of cells develops when chronic-phase progresses to blast crisis, we analyzed the DNA of six patients in both chronic phase and blast crisis.

### Table 3. Clinical Characteristics at the Time of Diagnosis of Ph'-Positive CML Patients With bcr Deletions

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Spleen (cm)</th>
<th>Hemoglobin* (g/dL)</th>
<th>WBC (x 10^3/L)</th>
<th>Platelets (x 10^3/L)</th>
<th>Basophils (%)</th>
<th>Time From Diagnosis to bcr Study (mo)</th>
<th>Duration of Chronic Phase (mo)</th>
<th>Karyotype at Time of bcr Study</th>
<th>Current Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63/M</td>
<td>+</td>
<td>N</td>
<td>114</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
<td>3</td>
<td>Ph', 95%</td>
<td>Died after undifferen-tiated BC</td>
</tr>
<tr>
<td>2</td>
<td>50/M</td>
<td>Tip</td>
<td>N</td>
<td>105</td>
<td>760</td>
<td>4</td>
<td>68</td>
<td>68</td>
<td>Ph', 100%</td>
<td>Died after myeloid BC</td>
</tr>
<tr>
<td>3</td>
<td>29/M</td>
<td>25 L</td>
<td>N</td>
<td>153</td>
<td>540</td>
<td>2</td>
<td>1</td>
<td>24+</td>
<td>Ph', 100%</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>4</td>
<td>33/M</td>
<td>O</td>
<td>N</td>
<td>34</td>
<td>854</td>
<td>1</td>
<td>6</td>
<td>42+</td>
<td>Ph', 95%</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>5</td>
<td>58/F</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>12</td>
<td>12+</td>
<td>Ph', 100%</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>6</td>
<td>32/M</td>
<td>N</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>84</td>
<td>84</td>
<td>84+</td>
<td>Ph', 100%</td>
<td>Accelerated phase</td>
</tr>
<tr>
<td>7</td>
<td>22/F</td>
<td>12 L</td>
<td>NA</td>
<td>1,300</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>9+</td>
<td>Ph', 100%</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>8</td>
<td>65/F</td>
<td>15 L</td>
<td>L</td>
<td>112</td>
<td>690</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>Clonal evolution†</td>
<td>Died due to undifferen-tiated BC</td>
</tr>
<tr>
<td>9</td>
<td>27/M</td>
<td>O</td>
<td>N</td>
<td>160</td>
<td>350</td>
<td>5</td>
<td>8</td>
<td>12+</td>
<td>Ph', 100%</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>10</td>
<td>29/M</td>
<td>20 L</td>
<td>291</td>
<td>336</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>Ph', 75%</td>
<td>Died after lymphatic BC</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available; BC, blast crisis.
* N, normal; L, low: male, <12 g/dL; female, <11 g/dL.
† Clonal evolution [46xx,t(3p-;4q+), t(9q+;22q-)], 55%: [46xx,t(9q+;22q-)], 40%. Hyperdiploid cells with additional Ph' and other changes, 5%.
ANALYSIS OF BREAKPOINTS WITHIN THE \emph{bcr} GENE

Table 4. Analysis of DNA for \emph{bcr} Rearrangement in Patients in Both the Chronic Phase and Blastic Crisis

<table>
<thead>
<tr>
<th>Sample From Chronic Phase</th>
<th>Sample From Blastic Crisis</th>
<th>Type of Blastic Crisis</th>
<th>Breakpoint Fragment</th>
<th>Additional Aberrant Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient No.</td>
<td>Time Diagnosis in Months</td>
<td>Diagnosis in Months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>61</td>
<td>Undifferentiated</td>
<td>F3</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>36</td>
<td>Lymphatic</td>
<td>F3</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>24</td>
<td>Myeloid</td>
<td>F2</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>48</td>
<td>Myeloid</td>
<td>F2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>11</td>
<td>Myeloid</td>
<td>F1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>Myeloid</td>
<td>F2</td>
</tr>
</tbody>
</table>

Only one patient showed an additional aberrant \emph{bcr} gene band. Bartram et al found a new chromosome rearrangement in one of four patients in blastic crisis\(^3\) and a deletion of \emph{bcr}/\emph{c-abl} sequences in another.\(^6\) However, such cases are rare among Ph'-positive CML patients. Despite the major development in our understanding of genetic events associated with CML pathogenesis, little is known about Ph'-positive CML evolution from a chronic to a more advanced stage. We cannot clearly demonstrate changes within the \emph{bcr-abl} gene to account for disease evolution in the majority of the cases we examined. However, this does not rule out (a) that more 5' secondary \emph{bcr-abl} changes are associated with disease evolution or that (b) minor changes (point mutations) not detected by the methodologies used by us contribute to disease progression. At this point, a more likely explanation is that activation of additional genes (on chromosomes 8, 22, and 17) cooperate with the \emph{bcr-abl} gene to lead toward a more aggressive disease stage.

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