CONCISE REPORT

Rearrangement of the bcr Gene in Philadelphia Chromosome-Negative Chronic Myeloid Leukemia


We studied the clinical, hematologic, cytogenetic, and molecular biologic features of seven patients with Philadelphia (Ph') chromosome-negative chronic myeloid leukemia (CML). In five cases the hematologic findings were indistinguishable from those of patients with classical Ph'-positive disease. Myeloid cells were studied by chromosome-bandng techniques. One patient had a masked Ph' chromosome (with translocation t(4;9;22)), one had a deletion involving chromosome 16, and one had a small minority population of 22q− cells without 9q+ but otherwise normal metaphases; metaphases from the other four patients were entirely normal. DNA prepared from the myeloid cells was digested with the restriction enzymes EcoRI, HindIII, BamHI, and BglII. Southern analysis using a 0.6-kb fragment of the breakpoint cluster region (bcr) gene showed the presence in each patient's DNA of a germline fragment together with a rearranged fragment or fragments with at least one of the restriction enzymes. We conclude that genomic changes in the bcr gene characteristic of CML can be present in the absence of a Ph' chromosome. © 1986 by Grune & Stratton, Inc.

THE MAJORITY of patients with chronic myeloid leukemia (CML) have a characteristic deletion of a portion of the long arm of one chromosome 22, the Philadelphia (Ph') chromosome, in their myeloid cells. The missing material is translocated to chromosome 9 such that the usual karyotype is described as t(9;22)(q34;q11). In Ph'-positive CML the oncogene (c-abl) normally present on chromosome 9 is translocated to chromosome 22 where it comes into juxtaposition with the 5' portion of a specific sequence of DNA named the breakpoint cluster region (bcr). A chimeric c-abl-related mRNA has been identified in cells from patients with CML and is associated with the presence of a fusion protein that has tyrosine kinase activity. Thus patients with Ph'-positive CML show evidence of rearrangement of DNA within the bcr region.

About 10% of patients regarded on clinical and hematologic grounds as having CML lack the Ph' chromosome. Some of these have a leukemia that is totally indistinguishable from Ph'-positive disease in all other respects, whereas in other cases subtle clinical or hematologic features suggest that the disease is different. We report results of studying the clinical, hematologic, cytogenetic, and molecular biologic features in seven patients with Ph'-negative CML.

MATERIALS AND METHODS

Patients. We selected for study seven patients who had attended the Hammersmith Hospital within the last 2 years with a diagnosis of Ph'-negative CML. Their clinical and hematologic features at diagnosis are shown in Table 1.

Granulocyte-macrophage progenitor cell (CFU-GM) assay. Mononuclear cells from the peripheral blood of five of the seven patients were cultured in a standard methylcellulose system for CFU-GM with mixed lymphocyte culture-conditioned medium as the source of colony-stimulating activity. Colonies consisting of 50 or more cells were scored after incubation for 12 days.

Cytogenetic analysis. Cytogenetic studies were carried out on fresh bone marrow cells or on fresh or cryopreserved buffy coat cells collected from the patients at the time of diagnosis. Chromosomes were prepared according to standard methods and analyzed after Giemsa banding.

bcr gene rearrangement. Leukemic cells were isolated from the peripheral blood or marrow samples, and high-molecular weight DNA was sheared by ultrasonic treatment.

Table 1. Clinical and Hematologic Details of Seven Patients With Ph'-Negative CML

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Spleen* (cm)</th>
<th>Age (yr)</th>
<th>Hb (g/dL)</th>
<th>WBC (x 109/L)</th>
<th>Platelets (x 109/L)</th>
<th>Differential (Percentage of 300 Cells)</th>
<th>CFU-GM per 10^6 Cells (x SEM)</th>
<th>Treatment and Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>25</td>
<td>24</td>
<td>8.3</td>
<td>174</td>
<td>941</td>
<td>6 6 21 44 6 14 0 4 0</td>
<td>360 ± 48</td>
<td>HU, PR</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>58</td>
<td>47</td>
<td>10.6</td>
<td>170</td>
<td>53</td>
<td>1 3 28 61 0 0.5 0.5 6 0</td>
<td>330 ± 30</td>
<td>HU, PR</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>31</td>
<td>65</td>
<td>12.1</td>
<td>46</td>
<td>375</td>
<td>0 0 10 56 17 2 1 1 14 0</td>
<td>340 ± 80</td>
<td>BU, HU, CR, BMT</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>53</td>
<td>60</td>
<td>10.5</td>
<td>77</td>
<td>492</td>
<td>0.5 14 67 2 1 7 85 0</td>
<td>0 ND</td>
<td>BU, HU, CR, BMT</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>62</td>
<td>50</td>
<td>10.6</td>
<td>105</td>
<td>87</td>
<td>0 4 31 58 0 0 1 2 2</td>
<td>ND ND</td>
<td>SRT, CR</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>43</td>
<td>58</td>
<td>10.3</td>
<td>506</td>
<td>319</td>
<td>2 4 28 59 2 3 1 1 1 1</td>
<td>324 ± 105</td>
<td>BU, CR</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>64</td>
<td>52</td>
<td>7.0</td>
<td>163</td>
<td>545</td>
<td>2 4 22 58 2 9 1 1 1 1 0.3</td>
<td>0.100 ± 425</td>
<td>BU, HU, CR, BMT</td>
</tr>
</tbody>
</table>

Table 1. Clinical and Hematologic Details of Seven Patients With Ph'-Negative CML

Abbreviations: Hb, hemoglobin; HU, hydroxyurea; BU, busulfan; SRT, splenic irradiation; CR, complete hematologic response; PR, partial hematologic response; BMT, bone marrow transplantation; NA, not available; ND, not determined; NAP. Neutrophil alkaline phosphatase (normal range, 30 to 100 units); BI, blasts; Pm, promyelocytes; My, myelocytes; Met, metamyelocytes; Neut, neutrophils; Eos, eosinophils; Baso, basophils; Mono, monocytes; Lymph, lymphocytes; NRBC, nucleated RBC.

* Spleen size was measured in cm below left costal margin.
†Two patients had blood films not typical of Ph' positive CML.

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DNA was prepared by standard procedures. All samples of DNA were digested with the restriction enzymes BamHI, BglII, EcoRI, and HindIII, separated on a 0.8% agarose gel by electrophoresis, and transferred to Hybond-N (Amersham, Buckinghamshire, UK) by the Southern technique. The filters were prehybridized and then hybridized at 43 °C with radiolabeled (\(^{32}\)P) probe. The filters were washed twice for 60 minutes in 0.1 x SSC (sodium chloride and sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate at 43 °C and autoradiographed for three to five days at -70 °C.

The 0.6-kb intron fragment (bcr-G) was subcloned from a commercially available bcr probe (Oncogene Sciences, Mineola, NY) and was labeled by the oligonucleotide priming method to a specific activity of 1 to 3 \times 10^{6} \text{cpm/ug} (Fig 1).^{9}

**RESULTS**

The hematologic findings for the seven patients are shown in Table 2. Their leukocyte counts ranged from 46 to 506 \times 10^{9}/L at diagnosis. The neutrophil alkaline phosphatase

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**Table 2. Cytogenetic Findings in the Seven Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Source of Cells</th>
<th>Number of Metaphases</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB</td>
<td>38</td>
<td>46, XY</td>
</tr>
<tr>
<td>2</td>
<td>BM</td>
<td>30</td>
<td>46, XY, del (22)(pter--q11::)</td>
</tr>
<tr>
<td>3</td>
<td>BM*</td>
<td>30</td>
<td>46, XY</td>
</tr>
<tr>
<td>4</td>
<td>PB</td>
<td>20</td>
<td>46, XX</td>
</tr>
<tr>
<td>5</td>
<td>BM*</td>
<td>20</td>
<td>46, XY</td>
</tr>
<tr>
<td>6</td>
<td>PB</td>
<td>25</td>
<td>46, XX</td>
</tr>
<tr>
<td>7</td>
<td>BM</td>
<td>30</td>
<td>46, XX, t(4;9;22)</td>
</tr>
</tbody>
</table>

*Except in two cases peripheral blood (PB) or bone marrow (BM) for these studies was collected before any treatment was administered. Marrow cells from patients 3 and 5 were studied after the patients had been treated with chemotherapy and splenic irradiation, respectively.*
score was low in the five patients studied at diagnosis. Two of the patients were thrombocytopenic. In each case the bone marrow in aspirate smears, trephine sections, or in both was hypercellular and showed gross granulocytic hyperplasia with maturation and no excess of blast cells. On morphologic grounds five patients (nos. 1, 2, 3, 6, and 7) were regarded as having a leukemia indistinguishable from Ph'-positive CML.

In two cases, however, the hematologic features were inconsistent with Ph'-positive CML. Patient 4 had many hypogranular neutrophils, a high percentage and a low basophil count; patient 5 also had gross morphologic abnormalities in the granulocyte series, including hypogranular or agranular myelocytes, metamyelocytes, and neutrophils, and he also lacked basophilia. In five of the seven cases, treatment usually effective in Ph'-positive CML was effective in restoring the patients' blood counts to normal; in two cases (nos. 1 and 2) only partial responses were observed. All seven patients were alive at the time of writing. Two patients (nos. 3 and 4) had been treated by allogeneic bone marrow transplantation and were well 2 and 8 months posttransplant, respectively.

The mean number of CFU-GM for the five patients studied was 1,091 ± 752 (SEM) per 10⁶ nucleated cells plated (range, 324 to 4,100) (Table 1). The corresponding mean values for ten patients with untreated Ph'-positive CML and for five normal subjects were 947 ± 171 (range, 298 to 2,114) and 9 ± 3 (range, 2 to 19), respectively.

The results of cytogenetic studies are summarized in Table 2. Patient 7 had a complex translocation, and her karyotype was otherwise normal. A third patient (no. 1) had a minority population of myeloid metaphases that showed 22q− (but no 9q+), but the majority of metaphases were normal. The other four patients had entirely normal karyotypes.

Southern blots prepared from leukemic cells and probed with the bcr-O probe showed a germline together with an abnormal restriction band in digests with one or more restriction enzymes in all seven cases (Table 3, Fig 1). Germline bands only were seen in DNA from the blood of seven normal subjects (data not shown).

**DISCUSSION**

The definition of Ph'-negative CML is necessarily imprecise because there is no single feature on which the diagnosis can be firmly based. In general, Ph'-negative CML patients differ from Ph'-positive patients in having a smaller spleen at corresponding leukocyte counts, more commonly thrombocytopenia at presentation, and a leukocyte differential that may lack a peak of myelocytes, may lack basophilia or eosinophilia, or may show monocytosis. There may also be dysplastic changes in the granulocyte series, which are rarely or never seen in Ph'-positive disease. Five of the patients reported in this paper had a form of CML that was identical with Ph'-positive disease, but in two (nos. 4 and 5), some features were atypical. In the five patients whose blood was cultured, the numbers of CFU-GM were in the range seen in Ph'-positive CML and very different from those seen in chronic myelomonocytic leukemia or in normal subjects. Four of the seven patients had normal karyotypes. The three other patients had various chromosomal abnormalities, but none had the classic t(9;22).

In one recent retrospective analysis of the hematologic features of 25 patients previously regarded as having Ph'-negative CML, the authors concluded that all but one case could be better reclassified either as examples of the myelodysplastic syndrome or as chronic myeloproliferative disorders other than CML. Essentially the same conclusion was reached in a retrospective study of 24 patients seen at the Mayo Clinic; the authors doubted the existence of Ph'-negative CML as a real entity. We believe, however, that Ph'-negative CML is a heterogeneous group within which a minority of cases are indistinguishable on clinical and hematologic criteria from Ph'-positive disease.

We found evidence of rearrangement in the bcr region in all seven patients studied in this series. These results appear to contrast with those of Morris et al who reported evidence for bcr rearrangement in only two of their five Ph'-negative patients. However, the three patients in that series without detectable bcr rearrangement were reclassified as having other forms of myeloproliferative disease. It is worth noting that we have also studied three other patients, not included in this series, with hematologic features consistent with chronic myelomonocytic leukemia; none had evidence of bcr rearrangement. In another recent report bcr rearrangement was found only in one of ten Ph'-negative patients; results of using only one restriction enzyme were presented, and different results might have been obtained if more enzymes had been used.

We cannot at present say whether the c-abl gene has come into juxtaposition with any portion of the bcr gene in these Ph'-negative cases, and we do not know whether the rearranged bcr gene is or is not retained on chromosome 22. abl-related genetic material was demonstrated on chromosome 22 in one of the cases reported by Morris et al. Moreover, Bartram and his colleagues described a patient with clinically typical CML who had a complex chromosomal translocation, t(9;12;22); cytogenetic and molecular biologic studies suggested that the bcr-abl hybrid gene was present but was located on chromosome 12 rather than on chromosome 22 as in classic Ph'-positive CML. However, in another case of Ph'-negative CML, bcr rearrangement was demonstrated without apparent translocation involving c-abl or expression of the novel abl-related mRNA species. Thus,
the extent to which c-abl is involved with the bcr rearrangement in Ph'-negative CML is not yet clear.

In conclusion, it seems that involvement of the bcr gene is a characteristic feature of CML, whether or not the karyotype is abnormal. It is clear, however, that clinically and hematologically the Ph'-negative cases form a heterogeneous group, and the full details of the molecular basis for the variation remain to be worked out.

REFERENCES
Rearrangement of the bcr gene in Philadelphia chromosome-negative chronic myeloid leukemia

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