Philadelphia Chromosome-Negative Chronic Myelogenous Leukemia Without Breakpoint Cluster Region Rearrangement: A Chronic Myeloid Leukemia with a Distinct Clinical Course

By Razelle Kurzrock, Hagop M. Kantarjian, Mordechai Shtalrid, Jordan U. Gutterman, and Moshe Talpaz

The hallmarks of chronic myelogenous leukemia (CML) include the Philadelphia chromosome (Ph) translocation ([t(9;22)(q34;q11)] and consistent molecular genetic aberrations: a break within a restricted 5.8 kb DNA segment, bcr, on chromosome 22q11; transposition of the c-abl proto-oncogene from chromosome 9q34 to 22q11; and formation of a hybrid bcr-abl protein with augmented tyrosine kinase enzymatic activity. These molecular phenomena may occur even in the absence of cytogenetic evidence of the Ph translocation. They are highly specific and sensitive markers for CML, and are presumed to play a significant role in the pathogenesis of this malignancy. Surprisingly, we have encountered 11 patients who lacked the Ph translocation, bcr rearrangement, and (in the four patients with available mRNA) a bcr-abl message, and yet had a disease phenotype analogous to that at diagnosis that was a morphologic facsimile of classic chronic phase CML. These patients presented with high white blood cell counts, neutrophilia, occasional basophilia, splenomegaly, and a hypercellular bone marrow with granulocytic hyperplasia and a left shift in myeloid maturation. Despite the striking resemblance between the early stages of bcr-negative and bcr-positive CML, disease progression manifests distinctly in these two disorders. In contrast to the blastic transformation that inevitably complicates bcr-positive CML, the natural history of our 11 Ph-negative, bcr-negative CML patients was characterized by increasing leukemia burden with leukocytosis, pronounced organomegaly, extramedullary infiltrates, and eventual bone marrow failure (anemia and thrombocytopenia) without marked increases in blast cells. Our current observations suggest that a chronic myeloid leukemia process can develop without associated changes in the bcr or c-abl genes. Although the initial phase of this disease is indistinguishable from CML, the presence or absence of molecular markers may aid in the prediction of the clinical course of Ph-negative CML.

Our laboratory, as well as other investigators, has described Ph-negative CML patients in whom bcr rearrangement was observed; indeed, a total of 48 patients with Ph-negative, bcr-positive CML have been reported in the world literature. The true incidence of this entity is difficult to determine because of patient selection. However, based on the experience at M.D. Anderson Cancer Center, approximately 5% of the total number of individuals referred with a confirmed diagnosis of CML have bcr-positive disease. Despite the absence of a chromosome 22 abnormality detectable by cytogenetic techniques. The pathognomonic bcr-abl mRNA and p210bcr-abl have been observed in all the samples derived from Ph-negative, bcr-positive CML patients that we examined. Further, the disease characteristics, response to therapy, and outcome of these patients are indistinguishable from that of individuals with Ph-positive CML. Therefore, the molecular fingerprint and clinical phenotype of bcr-positive CML is identical in Ph-positive and Ph-negative patients.

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The question arises as to whether all Ph-negative patients with classic CML will exhibit bcr rearrangement. Intuitively, we have felt that this should be true. Even so, we have now encountered 11 patients with clinical features highly suggestive of chronic phase CML, who have neither a discernible bcr rearrangement nor a bcr-abl message. In this report, we describe in detail the clinical, laboratory, and molecular characteristics of the Ph-negative, bcr-negative CML disease state.

MATERIALS AND METHODS

**Patient population.** All patients referred to UT M. D. Anderson Cancer Center with a tentative diagnosis of CML had a full work-up, including blood, bone marrow, and molecular studies, at our center. Informed consent was obtained according to institutional guidelines. Criteria for diagnosis of CML were: (1) a hypercellular bone marrow with granulocytic hyperplasia and a shift to the left in myeloid maturation; (2) persistent, unexplained, peripheral granulocytic leukocytosis of ≥20 × 10⁹ cells/L; and (3) absence of monocytois, polycythemia, and significant bone marrow myelofibrosis or dysplasia.

**DNA analysis.** High molecular DNA was prepared as described.¹ Fifteen micrograms of DNA was digested with restriction endonucleases in conditions recommended by the supplier (International Biotechnologies, Inc, New Haven, CT), electrophoresed on 0.8% agarose gel, blotted, and hybridized according to the method of Southern.² The probes were labeled by oligo primer extension to a specific activity of 1 to 3 × 10⁹ cpm/μg of DNA.³ After hybridization, filters were washed at 220 x 0.15 mol/L sodium chloride + 0.1% sodium dodecyl sulfate (SDS). Filters were then dried and autoradiographed.

**RNA analysis.** Total cellular RNA was isolated by a modified guanidinium isothiocyanate-cesium chloride method.⁴ Polyadenylated RNA was selected by chromatography on oligo (dT)-cellulose columns.⁵ The polyadenylated RNA (5 μg per lane) was size-fractionated by electrophoresis in 1.1% agarose gels containing 2.2 mol/L formaldehyde and blotted with 20 × SSC. The filters were hybridized to ³²P -radiolabeled DNA probes, washed, and radiographed in the same conditions as DNA filters.

**Probes.** A 3' bcr (1.2 Kb HindIII/BglII genomic probe [bcr (PR-1)]) and a larger "universal" bcr probe encompassing most of the 5.8 kb bcr region (Ph1/bcr-3) (Oncogene Science, Inc, Manhasset, Long Island, NY; Fig 1) were used to determine bcr rearrangement status. A 5' EcoRI/Pst I fragment of the bcr gene⁶ and an EcoRI/BamHI fragment derived from the human c-abl gene⁷ were used to detect RNA after Northern blotting.

**Cytogenetic analysis.** Chromosome studies were performed on bone marrow cells from all patients at the time of diagnosis or referral and every 3 to 6 months during follow-up. A minimum of 20 metaphases were analyzed from each sample, and the karyotype was reported according to the International System for Human Cytogenetic Nomenclature.⁸ The bone marrow samples were cultured overnight without mitogenic stimulating in Ham's F10 medium supplemented with 10% fetal calf serum. Standard cytogenetic procedures were used on the samples, and the slide preparations were stained with Giemsa after a trypsin pretreatment, which yielded G-banded chromosomes.

**RESULTS**

**Cytogenetic analysis.** No patient had evidence of a chromosome 9 or 22 abnormality. Six patients had a normal diploid karyotype; five patients, an abnormal karyotype (Table I). In the five patients with chromosome aberrations, the abnormal clone was seen in only a fraction (10% to 57%) of the metaphases. Further, clonal evolution was observed in only two patients, both of whom already showed karyotype abnormalities at the time of diagnosis. Chromosomal aberrations involved chromosome 21 (three patients), and chromosomes 1, 8, 13, 15, 17, 19, 20, and X in each of one patient (Table I).

**DNA analysis for bcr rearrangement.** Southern blot analysis was initially performed after DNA digestion with four restriction enzymes (BamHI, BglIII, EcoRI, and HindIII) and hybridization with the 3' bcr probe (bcr [PR-1]). All filters were then rehybridized with the universal Ph1/bcr-3 probe. Because the latter probe incorporates most of the 5.8 kb bcr, it allows detection of bcr rearrangement in patients with deletions in parts of the bcr, virtually eliminating the possibility of false-negative results. Using these techniques, bcr rearrangement was not discerned in any patient (Fig 2).

**mRNA analysis.** To rule out the remote possibility that a unique mechanism of genomic reorganization might result in production of a bcr-abl message, despite the absence of bcr rearrangement, we performed Northern blotting on mRNA derived from four of our Ph-negative, bcr-negative CML.

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Fig 1. Restriction enzyme map of human 5.8 kb bcr segment, within a map of a portion of the bcr gene (adapted from Heisterkamp et al). Restriction enzymes: BamHI, B; BglII, Bg; HindIII, H; EcoRI, E; XbaI, X; and SstI, S. Exons are indicated by black boxes below the map. The bcr probes used in the current study are shown below the map.
Table 1. Cytogenetic and Molecular Parameters

<table>
<thead>
<tr>
<th>Patient</th>
<th>Metaphases</th>
<th>Karyotype</th>
<th>Clonal Evolution*</th>
<th>bcr Rearrangement†</th>
<th>mRNA</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>20 (100)</td>
<td>46,XY</td>
<td>N</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>22 (88)</td>
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<tr>
<td></td>
<td>1 (4)</td>
<td>47,XY, + 8</td>
<td></td>
<td>4.5 and 6.7 kb bcr mRNAs</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>46,XY</td>
<td>N</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>17 (89)</td>
<td>46,XY</td>
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<td>N</td>
<td>6.0 and 7.0 kb abl mRNAs</td>
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<tr>
<td></td>
<td>2 (11)</td>
<td>47,XY, + 21</td>
<td></td>
<td>4.5 and 6.7 kb bcr mRNAs</td>
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</tr>
<tr>
<td>5</td>
<td>18 (90)</td>
<td>46,XY</td>
<td>Y; at 32 mo new clone appeared = 48,XY, + X,15q−, + 21</td>
<td>6.0 and 7.0 kb abl mRNAs</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>48,XY, + X,15q−, + 21</td>
<td></td>
<td></td>
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<td>N</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>13 (43)</td>
<td>46,XX</td>
<td>N</td>
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</tr>
<tr>
<td>9</td>
<td>17 (57)</td>
<td>46,XX,t(1p−;13q+)</td>
<td>6.0 and 7.0 kb abl mRNAs</td>
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<td>15 (79)</td>
<td>46,XY</td>
<td>Y; at 31 mo all metaphases = 46,XY,20q−</td>
<td>4.5 and 6.7 kb bcr mRNAs</td>
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<td>46,XY</td>
<td>N</td>
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<td>ND</td>
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</tbody>
</table>

Values for cytogenetic analysis and molecular data are from either the time of diagnosis or the time of referral to M.D. Anderson Cancer Center. Abbreviations: N, no; Y, yes; ND, not done.

*Bone marrow metaphases were analyzed every 3 to 6 months for changes in karyotype.
†DNA was digested with BglII, BamHI, EcoRI, and HindIII restriction enzymes, analyzed on Southern blots, and hybridized with both a 3′ (bcr [PR-1]) bcr probe and the universal Phil/bn-3 probe encompassing most of the 5.8 kb bcr segment.

patients (Fig 3). The 8.5 kb aberrant bcr-abl transcript was not observed when the filters were probed with either the c-abl or the bcr probe. The normal 4.5 and 6.7 kb bcr transcripts (not shown), as well as the normal 6.0 and 7.0 kb abl transcripts (Fig 3), were consistently seen (Table 1).

Disease phenotype. The clinical features and laboratory parameters of the 11 Ph-negative, bcr-negative patients are summarized in Tables 2 and 3. There were seven men and four women. Their median age was 60 years (range, 46 to 70 years). All patients had splenomegaly. At the time of diagnosis, the median white blood cell (WBC) count was 45 × 10⁹ cells per liter (range, 23 to 300 × 10⁹ cells per liter); median platelet count, 295 × 10⁹ cells per liter (range, 50 to 1,046 × 10⁹ cells per liter). All patients had significant granulocytosis. A small percentage of immature myeloid cells (bands, metamyelocytes, and myelocytes) were also seen in the peripheral blood of all patients. Eosinophilia was not observed. Four patients had peripheral blood basophilia ± 3%. Polycythemia was not observed in any patient. Seven patients were anemic at diagnosis. Six patients had low LAP scores. Bone marrow cellularity was greatly increased (median cellularity = 100%) with granulocytic hyperplasia, a left shift, and an elevated myeloid:erythroid ratio in all patients. Light microscopic examination of bone marrow morphology was consistent with a diagnosis of chronic phase CML in each case. CML is nosologically related to other myeloproliferative disorders such as idiopathic myelofibrosis, polycythemia vera, and essential thrombocytopenia. How-

![Southern blot analysis of DNA samples. DNA was digested with restriction enzyme EcoRI. Blots were hybridized with the 3′ bcr (bcr[PR-1]) probe (Fig 1). Lanes 1-11, DNA from patients 1 to 11, respectively (Table 1); only the germ-line bcr band is seen. Lane 12, DNA from a normal volunteer; only the germline bcr band is seen. Lane 13, DNA from a Ph-positive CML patient; a germline and a rearranged band are seen.](image)
ever, the exuberant marrow fibrosis, expanded red cell mass, and florid megakaryocytic hyperplasia that form the respective hallmarks of these diseases were not seen in our patients. In addition, Ph-negative CML has, at times, been confused with certain myelodysplastic disorders; careful review of the bone marrow slides of our patients failed to reveal significant cellular dysplasia.

Response to therapy. All patients were treated. Complete hematologic remission was defined as achievement of a WBC count of \(< 9 \times 10^9\) cells per liter with a normal...
eight patients with progressive disease have developed evidence of bone marrow failure, manifested by increasing bone marrow basophilia (basophils respectively) occurred in two patients. Significant bone marrow cells), Sweet's syndrome (neutrophilic dermal infiltrates at two patients who were splenectomized. One patient received alternatively, patients who attained normal blood counts but had a persistently abnormal differential or residual splenomegaly were also classified as having a partial remission. Partial remission referred to achievement of a WBC count x 10^9/L and no evidence of splenomegaly. Partial remission is characterized by a pronounced peripheral blood granulocytosis and an increased myeloid compartment in a hypercellular bone marrow. The chronic phase lasts about 3 to 4 years, evolution, wherein the leukemic cells lose their capacity for differentiation. An accelerated phase of approximately 6 months’ duration ensues, followed by a rapidly terminal blastic crisis phase. During these final stages of CML, a marked increase in blast cells occurs, reminiscent of that observed in acute leukemias.

DISCUSSION Ph-positive CML is a clonal myeloproliferative disorder resulting from oncogenic transformation of a bone marrow stem cell. In its early or chronic stage, the disease phenotype is characterized by a pronounced peripheral blood granulocytosis and an increased myeloid compartment in a hypercellular bone marrow. The chronic phase lasts about 3 to 4 years, but inevitably undergoes a seemingly preordained pattern of evolution, wherein the leukemic cells lose their capacity for differentiation. An accelerated phase of approximately 6 months’ duration ensues, followed by a rapidly terminal blastic crisis phase. During these final stages of CML, a marked increase in blast cells occurs, reminiscent of that observed in acute leukemias.

In recent years, significant advances have been made toward understanding the pathogenesis of CML. At the molecular level, Ph-positive CML is characterized by bcr differential and no evidence of splenomegaly. Partial hematologic remission referred to achievement of a WBC count ≤20 x 10^9 cells per liter and a ≥50% decline in counts; alternatively, patients who attained normal blood counts but had a persistently abnormal differential or residual splenomegaly were also classified as having a partial remission.

All nine patients treated with hydroxyurea achieved a partial remission. One patient received busulphan, and had a partial remission. There was no disease improvement in the two patients who were splenectomized. One patient received interferon gamma but did not respond. Two of six patients who received recombinant interferon alpha have achieved a complete hematologic remission. The responses are ongoing at 9+ and 40+ months.

Clinical course and prognosis. Disease progression has been characterized by massive organomegaly (splenomegaly ± hepatomegaly) and extramedullary manifestations of leukemia, including chloromas (skin accumulations of blastic cells), Sweet’s syndrome (neutrophilic dermal infiltrates accompanied by fever), lymphadenopathy, and ascites (Table 2). In contrast to the situation in Ph-positive CML, blastic transformation did not occur. Indeed, only one patient ever showed greater than 15% blasts in the bone marrow (patient 1, 18% blasts), and most patients had 0 to 10% blasts, despite other evidence of disease progression. Marked bone marrow basophilia (basophils = 11% and 31%, respectively) occurred in two patients. Significant bone marrow fibrosis complicated the course of only one patient. Six of eight patients with progressive disease have developed evidence of bone marrow failure, manifested by increasing anemia and thrombocytopenia. Concomitantly, a rise in WBC count with an attenuated response to therapy has been generally observed.

The median survival for our Ph-negative, bcr-negative CML patients has not been reached. At present, the median follow-up period is 24 months (range, 8 to 57 months). Analysis by the method of Kaplan and Meier predicts about a 50% survival at 37 months in this group. Seven patients have died at 8, 12, 15, 24, 37, 46, and 57 months, respectively, after diagnosis. Death has been attributed to increasing, uncontrollable organomegaly, hemorrhage associated with thrombocytopenia, and infection.

DISCUSSION Ph-positive CML is a clonal myeloproliferative disorder resulting from oncogenic transformation of a bone marrow stem cell. In its early or chronic stage, the disease phenotype is characterized by a pronounced peripheral blood granulocytosis and an increased myeloid compartment in a hypercellular bone marrow. The chronic phase lasts about 3 to 4 years, but inevitably undergoes a seemingly preordained pattern of evolution, wherein the leukemic cells lose their capacity for differentiation. An accelerated phase of approximately 6 months’ duration ensues, followed by a rapidly terminal blastic crisis phase. During these final stages of CML, a marked increase in blast cells occurs, reminiscent of that observed in acute leukemias.

In recent years, significant advances have been made toward understanding the pathogenesis of CML. At the molecular level, Ph-positive CML is characterized by bcr...
rerrangement and production of an enzymatically-enhanced, CML-specific, marker protein, p210bcr-ab1. In this regard, we have previously examined DNA derived from 100 patients with Ph-positive CML, and found all to have discernible rearrangements when two bcr probes were used. In addition, bcr rearrangement and/or expression of a bcr-abl product has never been detected in any normal tissue nor in any disorder other than Ph-positive leukemias. Therefore, our current study's strategy of using two bcr probes and molecular techniques for assessing bcr status is highly sensitive and specific for CML.

Based on the premise that bcr rearrangement and p210bcr-ab1 are essential as initiating events in the development of CML, we were at first convinced that all cases of CML would be bcr-positive. Indeed, it is well-established that even CML patients lacking cytogenetic evidence of Ph may have subchromosomal molecular changes identical to those seen in Ph-positive patients. The clinical outcome of these Ph-negative, bcr-positive patients is identical to that of Ph-positive patients, underscoring the importance of bcr-abl in the pathogenesis of the CML phenotype. We were therefore surprised to encounter individuals with CML who lacked bcr rearrangement. In this report, we characterize seven men and four women with classic features of chronic phase CML, who have neither cytogenetic nor molecular changes detectable on chromosomes 9 and 22, despite a meticulous search for a break within bcr and/or a bcr-abl message. These patients represented approximately 3% of the CML patients referred to our institute during the study period. They presented with high WBC counts, granulocytosis, basophilia, splenomegaly, and a hypercellular bone marrow morphology indistinguishable from that found in the typical case of bcr-positive CML. A wide range of platelet counts (50 to 1,046 x 10^9 cells per liter) was noted at diagnosis, though the 18% incidence of thrombocytopenia may be somewhat higher than that (5%) previously observed in our Ph-positive population. Treatment with hydroxyurea successfully controlled elevated WBC counts in a manner similar to that expected in their bcr-positive counterparts. Two of six patients (33%) have also shown a favorable response to interferon alpha, an agent effective in the management of newly-diagnosed, Ph-positive, chronic phase CML patients. The 33% remission rate in bcr-negative disease appears less than the 75% rate generally achieved in bcr-positive disease, but the small number of subjects treated in the current study precludes a statistically valid comparison. The 50% probability of survival of 37 months (Kaplan and Meier analysis) in our bcr-negative cases was similar to the 39 months previously established at our institution for Ph-positive CML patients. At presentation, individuals with Ph-negative, bcr-negative CML were older (median age = 60 years) than those with Ph-positive CML (median age = 46 years). This was the only distinction that could be firmly established between the two groups. Hence, it appears that, in a small subset of patients, the development of features of chronic phase CML is divorced from derangements in the bcr and c-abl genes.

If distinct molecular events can lead to similar disease states, their elucidation may enhance our understanding of the biology of the leukemic process. It is possible that the normal c-abl protein participates in the control of hematopoietic cell growth as part of a mitogenic pathway that includes a growth factor, a growth factor receptor, and an intracellular protein signal system. In bcr-positive CML, the formation of the aberrant p210bcr-ab1, a cytoplasmic protein with constitutively-augmented tyrosine phosphokinase activity (an enzymatic property known to regulate protein functions) may serve to circumvent the natural feedback control mechanisms of this system. It is then conceivable that the chronic phase CML state could develop in the absence of bcr-abl because of perturbations in other parts of the pathway.

The natural history of bcr-positive CML is typified by evolution from a chronic phase to a blast crisis stage. In the latter stage, the myeloid cell loses its capacity for terminal differentiation, and immature hematopoietic cells rapidly proliferate. It has been suggested that secondary genetic driving forces are necessary for progression of the disease. The presence of bcr-abl may promote the appearance of these secondary changes, yet assume subordinate importance for their maintenance once they are established. In support of these concepts, greater than 80% of Ph-positive CML patients develop evidence of clonal evolution—an additional Ph, trisomy of chromosome 8, and/or isochromosome 17q— that presages blastic metamorphosis. Further, Bartram et al has shown in one patient the persistence of blast crisis despite the molecular deletion of bcr-abl sequences. Our bcr-negative CML patients, while demonstrating a "classic" chronic phase of disease, showed neither progression to blastic transformation nor the clonal evolution pattern of Ph-positive CML. Instead, disease deterioration was characterized by increasing leukemia burden with burgeoning organomegaly and eventual bone marrow failure. Therefore, if bcr-abl in some way facilitates the genetic instability leading to the development of specific secondary cytogenetic aberrations and blastic transformation, the lack of bcr-abl would explain the dichotomy in manifestations of progressive disease in bcr-negative as compared with bcr-positive CML patients.

In summary, our findings indicate that a Ph-negative, bcr-negative, chronic phase CML state exists. At the time of diagnosis, patients with this disorder are not clinically distinguishable from those with bcr-positive CML. However, because the course of bcr-negative CML is distinct from that of its bcr-positive counterpart, investigation of bcr status should be part of the work-up of individuals with Ph-negative CML. Furthermore, this subset of patients merits additional study, as they may hold important clues to the biology of chronic myeloid leukemogenesis.

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