Heterogeneity in Lineage Derivation of Philadelphia-positive Acute Lymphoblastic Leukemia Expressing \( p^{190BCR-ABL} \) or \( p^{210BCR-ABL} \): Determination by Analysis of Individual Colonies with the Polymerase Chain Reaction

Zeev Estrov, Moshe Talpaz, Hagop M. Kantarjian, Theodore F. Zipf, Kenneth L. McClain, and Razelle Kurzrock

Section of Biologic Studies, Department of Clinical Investigation, and Divisions of Medicine, Pediatrics, and Laboratory Medicine, University of Texas M. D. Anderson Cancer Center and Baylor College of Medicine, Texas Children's Hospital, Houston, Texas 77030

**ABSTRACT**

The molecular hallmark of Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL) is the expression of 1 of 2 alternate forms of the aberrant BCR-ABL protein—\( p^{210BCR-ABL} \) or \( p^{190BCR-ABL} \). The presence of BCR-ABL message provides a target for analyzing the lineage derivation of this disease. We, therefore, studied myeloid and erythroid progenitor involvement in Philadelphia chromosome-positive ALL. Bone marrow low-density cells from Philadelphia chromosome-positive ALL patients (5 with the \( p^{190BCR-ABL} \) and 2 with the \( p^{210BCR-ABL} \) abnormality) were cultured in the mixed colony culture assay. cDNA from individually plucked colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies was then analyzed using the hybridization protection assay in conjunction with the polymerase chain reaction to detect BCR-ABL molecular aberrations. Colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies from 1 of 5 \( p^{190BCR-ABL} \)-positive patients and 1 of 2 \( p^{210BCR-ABL} \)-positive patients expressed BCR-ABL transcripts, whereas colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies from the other patients did not. Our study suggests that the origin of both \( p^{190BCR-ABL} \) and \( p^{210BCR-ABL} \)-positive ALL is heterogenous with involvement of either a pluripotent precursor or a lymphoid lineage-committed hematopoietic progenitor.

**INTRODUCTION**

Ph+ ALL is a malignant disorder in which treatment is only partially effective, and the prognosis is considerably worse than in ALL lacking the Philadelphia chromosome (1, 2). The relationship between this disease and Ph+ CML has been the subject of significant speculation (3), and, because of the poor prognosis and the presence of the Ph abnormality, the question of multipotential stem cell involvement in Ph+ ALL as in CML has arisen. Alternatively, the heterogeneous molecular aberrations found in Ph+ ALL may reflect the involvement of distinct target cells.

The Ph abnormality results from a reciprocal translocation between the long arms of chromosomes 9 and 22 \([\text{t}(9;22) (q34;q11)]\). This anomaly transposes the C-ABL gene from chromosome 9 to chromosome 22. A new hybrid gene called BCR-ABL is thus created. Molecular studies of the breakpoint on chromosome 22 have, however, revealed 2 distinct patterns in Ph+ ALL. In about 50% of adults and 10% of children with Ph+ ALL, the break occurs in the central region of the BCR gene (the major bcr), and transcription of BCR-ABL results in a hybrid 8.5-kilobase mRNA that codes for a Mr 210,000 protein \( (p^{190BCR-ABL};p^{210}) \) (4–8). In all patients producing \( p^{210BCR-ABL} \), the genetic configuration of BCR-ABL is identical to that found in Ph+ CML, i.e., either exon b2 or exon b3 of the bcr is coupled to C-ABL exon 2 \((b_2-a_2 \) or \( b_3-a_2 \) junction) (6). The first intron of the BCR gene is the site of the break on chromosome 22 in the other half of the adults and 90% of children with Ph+ ALL. A smaller 7.0-kilobase BCR-ABL fusion mRNA is produced and encodes a Mr, 190,000 protein \( (p^{190BCR-ABL};p^{190}) \) (8–11). In these patients, the first exon of the BCR gene is spliced to the second exon of the C-ABL gene \( (e_1-a_2 \) junction).

Current studies examining stem cell involvement in Ph+ ALL have been performed on only a very small number of patients (12–15). These investigations have suggested a multipotential stem cell origin in 4 of 5 adults and 2 of 2 children, because metaphases of individually removed CPU-erythroid, CPU-GM, and CPU-mixed, erythroid-granulocyte-macrophage were Ph+ (12, 13). However, the molecular subtypes of these leukemia cells were not evaluated. In a molecular-based study, Ph+ ALL with rearrangement within the 5.8-kilobase region of the bcr \((b_2-a_2 \) junction or \( b_3-a_2 \) junction; \( p^{210} \)-type abnormalities) showed multilineage involvement in 2 of 3 patients (14). Currently, we have used a new technique—HPA in conjunction with the PCR (1, 16, 17)—to detect BCR-ABL cDNA in individually plucked colonies from seven Ph+ ALL patients. In all CPU-GM and BFU-E colonies from 4 of 5 \( p^{190} \)-positive patients, the BCR-ABL molecular aberration was not expressed. Similarly, 1 of the 2 \( p^{210} \)-positive patients lacked BCR-ABL+ colonies. However, one patient with each of \( p^{190BCR-ABL} \) and \( p^{210BCR-ABL} \) disease demonstrated BCR-ABL-expressing CPU-GM and BFU-E colonies. Our observations indicate that, in ALL, both the \( p^{190BCR-ABL} \) and the \( p^{210BCR-ABL} \) anomaly can be generated in either a lineage-committed or in a multipotent precursor cell.

**MATERIALS AND METHODS**

Patients with a diagnosis of Ph+ ALL were the subjects of this study. Patients were required to have: (a) a documented Ph abnormality by karyotype analysis (18); (b) a new onset of ALL presentation without a preceding antecedent hematological disorder or CML; and (c) 30% or more marrow blasts. Approval for these studies was obtained from the Institution Review Board. Workup of the patients at presentation included a history and physical examination; complete blood, differential, and platelet counts; SMA 12, including hepatic and renal functions; bone marrow aspiration and biopsy for morphology, cytochemical, and enzymatic stains (myeloperoxidase, chloracetate, non-specific esterase, periodic acid-Schiff, Tdt); immunophenotyping (19, 20); karyotypic analysis (18); electron microscopy (21), and molecular studies as described below.

The classification of the acute leukemia was based on the French-American-British criteria and histochemical stains (22, 23). ALL was diagnosed if the blasts were morphologically lymphoid, myeloid-peroxidase-negative, and Tdt-positive and/or common acute lymphocytic leukemia antigen (CD10)-positive.

**CFU-Granulocyte, Erythroid, Monocyte, Macrophage Assay**

A modification of a previously described CFU-granulocyte, erythroid, monocyte, macrophage assay was used (24, 25). In brief, \( 2 \times 10^5 \) nucleated low-density bone marrow cells were cultured in 0.8% \((\nu+\nu) \) methylcellulose with Iscove’s modified Dulbecco’s medium (GIBCO, Grand Island, NY), 30% fetal calf serum,
10 ng/ml recombinant GM-colony-stimulating factor or 15 ng/ml IL-3, 50 ng/ml stem cell factor (Immunex Corp, Seattle, WA), and 1.0 units/ml human erythropoietin (British Columbia Cancer Research Institute, Vancouver, Canada). One ml of the culture mixture was plated in 35-mm Petri dishes (Nunc, Inc., Naperville, IL) and incubated at 37°C with 5% CO₂ in air in a humidified atmosphere in duplicate. All cultures were evaluated after 14 days for BFU-E colonies (defined as an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies), and CFU-GM colonies (defined as a cluster of 40 or more granulocytes, macrophages, or both). Individual colonies were plucked from the cultures with a micropipette for a further analysis.

**Cell Line Clonogenic Assay (26).** A CML erythroid blast crisis cell line (K562) and a Ph⁺ ALL cell line (ALL-1, kindly provided by Dr. G. Rovera, Wistar Institute) were plated in duplicate 35-mm Petri dishes (Nunc) at a concentration of 2 × 10⁴ cells/ml in 0.8% (v:v) methylcellulose in RPMI (GIBCO) with 10% fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO₂ with air. Cultures were evaluated at day 6 using an inverted microscope. A cluster of more than 40 cells was defined as a colony.

**Molecular Studies.** Bone marrow from Ph⁺ ALL patients was analyzed for production of mRNA encoding p190BCR-ABL or p210BCR-ABL with the use of the PCR and specific primers encompassing the mRNA splice junctions to amplify cDNA. The product was then detected with the use of HPA as described previously (16, 17) and as detailed below. Patients with anentity-a2 junction produced p190BCR-ABL (9-11); those with a b2-a2 or b3-a2 junction produced p210BCR-ABL (27). Individual colonies from patient samples were then analyzed. All samples were simultaneously coamplified for the normal ABL transcript (using primers encompassing ABL exons Iβ and II) to ensure the presence of intact cDNA and a successful amplification procedure.

To avoid false-positive results with the PCR technique due to potential contamination, the following precautions were undertaken: (a) the thermal cycler was kept in a separate laboratory from the room where cell collection, RNA processing, and cDNA synthesis were performed; (b) no amplified samples were brought back into the room where RNA processing was performed; (c) at least one negative control was run for each experiment; (d) bone marrow samples from each patient were run at least two different times; and (e) colonies from each patient were plucked, prepared, and amplified by 2 technicians, who worked totally independently.

**Bone Marrow Sample Preparation and PCR Amplification.** We used PCR to establish the splice junction in each patient analyzed. Total cellular RNA was extracted and cDNA prepared as described previously (28). RNA from K562 cells, a CML erythroid blast crisis cell line, and from patients with Ph⁺ CML, were used as positive controls for cDNA containing b2-a2 and b3-a2 junctions. The ALL-1 cell line was used as a positive control for the e1-a2 mRNA transcript (9, 10, 29). HL-60 (a Ph- myelocytic leukemia cell line) and normal human endometrial RNA were used as negative controls. One μg of total RNA from cell lines or 1% of the total RNA from 50 to 200 × 10⁶ WBC from patient samples was used for amplification reactions. The PCR amplification was performed for 40 cycles using previously described primers encompassing the b2-a2, b3-a2, and e1-a2 junctions (30). For amplifications of the normal ABL transcript, a primer encompassing ABL exon II (31) was used to ensure the presence of intact cDNA and a successful amplification procedure.

**PCR Amplification of Hematopoietic Colonies.** CFU-GM and BFU-E colonies from patient samples were plated from the tissue culture dishes with a Pasteur micropipette as described previously (31). To validate our procedure, the samples were brought back into the room where RNA processing was performed; (a) the thermal cycler was kept in a separate laboratory from the room where cell collection, RNA processing, and cDNA synthesis were performed; (b) no amplified samples were brought back into the room where RNA processing was performed; (c) at least one negative control was run for each experiment; (d) colonies from each patient were plucked, prepared, and amplified by 2 technicians, who worked totally independently.

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DISCUSSION

In the current study, we have applied a hybridization protection assay to detect PCR-amplified BCR-ABL cDNA from individual CFU-GM and BFU-E colonies. This technique provides a simpler and more rapid alternative to the more standard approach of resolving PCR products on agarose gels and confirmation of identity by Southern blotting (17). We found that CFU-GM and BFU-E colonies obtained from 4 of 5 Ph+ ALL patients who were p190+ did not show molecular evidence of involvement in the malignant process. Our observations are consistent with the notion that in some individuals with p190BCR-ABL+ ALL, the disease is lineage-restricted and does not involve the committed myeloid or erythroid precursors (i.e., CFU-GM and BFU-E) or a pluripotent stem cell. Several alternate explanations are conceivable, though unlikely. For instance, it is possible that the maturation of the leukemic myeloid precursors is "arrested" before the stage of CFU-GM or BFU-E, or that the leukemic precursors do not form colonies. Negating this explanation are our results demonstrating BCR-ABL transcripts in the CFU-GM and BFU-E colonies of one p190BCR-ABL+ ALL patient. One of 2 patients with p210-positive ALL also exhibited the BCR-ABL aberration in CFU-GM and BFU-E colonies. The latter results are consistent with a study by Turhan et al. (14), who demonstrated, by Southern blotting of DNA, that granulocytes in 2 of 3 p210+ acute leukemia patients with p210-positive ALL also exhibited the BCR-ABL aberration in CFU-GM and BFU-E colonies. We then examined the patients' marrow samples (Table 4). In patients 1 through 5, we detected the e1-a2 junction in bone marrow low-density cells, indicating that they produced p190BCR-ABL. Nevertheless, the e1-a2 junction was not detected in the CFU-GM and BFU-E colonies of patients 1, 2, 3, and 5. (Only colonies in which normal ABL was detected were considered evaluable, ruling out the possibility of inadequate or degraded RNA, or an unsuccessful amplification procedure.) In contrast, colonies obtained from Patient 4 showed BCR-ABL expression. With regard to the 2 p210BCR-ABL+ patients, 4 of 10 CFU-GM and BFU-E colonies from Patient 7 had a detectable b2-a2 junction; this was the junction that characterized the original leukemic clone. In Patient 6, however, neither b2-a2 nor b3-a2 junction was found in the colonies, though both junctions were discernible in the leukemic cells.

Table 2 BCR-ABL junctions in ALL-1 cell line (Ph+, p190BCR-ABL+)

<table>
<thead>
<tr>
<th>b2-a2 junction (p210)</th>
<th>b3-a2 junction (p210)</th>
<th>e1-a2 junction (p190)</th>
<th>Normal ABL (RLUs)</th>
<th>Normal ABL (RLUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell sample</td>
<td>4,926 (neg)</td>
<td>4,861 (neg)</td>
<td>568,934 (pos)</td>
<td>789,456 (pos)</td>
</tr>
<tr>
<td>Colony no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single colonies</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>e1-a2 junction (RLUs)</td>
<td>132,491 (pos)</td>
<td>231,457 (pos)</td>
<td>80,246 (pos)</td>
<td>88,369 (pos)</td>
</tr>
</tbody>
</table>

Table 3 BCR-ABL junctions in a CML patient and a normal volunteer

<table>
<thead>
<tr>
<th>Plucked colonies</th>
<th>Cells</th>
<th>Colony type</th>
<th>b2-a2 junction (p210)(RLUs)</th>
<th>b3-a2 junction (p210)(RLUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML patient</td>
<td>CFU-GM</td>
<td>2,542 (neg)</td>
<td>222,020 (pos)</td>
<td>677,184 (pos)</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>2,963 (neg)</td>
<td>52,223 (pos)</td>
<td>298,225 (pos)</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>4,310 (neg)</td>
<td>2,697 (neg)</td>
<td>2,001 (neg)</td>
</tr>
<tr>
<td>Normal volunteer</td>
<td>CFU-GM</td>
<td>2,375 (neg)</td>
<td>1,970 (neg)</td>
<td>2,237 (neg)</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>2,384 (neg)</td>
<td>2,001 (neg)</td>
<td>2,697 (neg)</td>
</tr>
</tbody>
</table>

* Neg, negative; pos, positive.

* R. Kurzrock, unpublished observations.
are actually CML blast crisis in a patient who eluded diagnosis before transformation. Because p210BCR-ABL occurs in the vast majority of CML patients, and because p190BCR-ABL is found in Ph+ acute leukemia but not generally in CML, several investigators have postulated that p190BCR-ABL+ acute leukemia represents a de novo leukemic process, whereas p210BCR-ABL+ acute leukemia represents CML blast crisis. Yet, in examining a series of 32 patients with Ph+ ALL, we found that the clinical manifestations, response to therapy, and morphology of p210+ ALL did not differ from those of p190+ ALL (8). Our current observations suggesting that p190-positivity is often not associated with a multilineage disorder are consistent with the concept that most patients bearing this molecular abnormality do not have CML in blast crisis; rather, they may have a true ALL.

Further, the finding that the disease in p210+ ALL patients may originate either in a multipotent stem cell (as in CML) or in a lineage-committed progenitor (as in most cases of ALL) suggests the possibility that only a subset of p210+ acute leukemia actually represents CML blast crisis. It may therefore be worthwhile to examine a larger number of individuals to ascertain whether the multilineage form of p210+ acute leukemia behaves differently from the lineage-committed form or from p190+ disease.

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REFERENCES
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