Chronic Myelogenous Leukemia: Amplification of a Rearranged c-abl Oncogene in Both Chronic Phase and Blast Crisis

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The specific genetic events that distinguish the blast crisis from the chronic phase cells of chronic myelogenous leukemia (CML) are unknown. The most common karyotypic change that occurs as CML evolves from chronic phase to blast crisis is the development of multiple Philadelphia (Ph1) chromosomes, each of which is presumably harboring a translocated c-abl oncogene. We describe here a patient with CML who presented in lymphoid blast crisis with three Ph1 chromosomes/metaphase associated with an amplified, rearranged c-abl oncogene fragment and high levels of the aberrant 8-kilobase bcr-abl transcript. This rearranged c-abl fragment was amplified to a similar degree in both the patient’s blast crisis cells and in his terminally differentiated granulocytes, but the level of the aberrant CML-specific bcr-abl transcript was some eight- to 16-fold higher in the blast crisis cells v the granulocytes. This analysis indicates that genomic amplification of a translocated c-abl oncogene, although perhaps important in the evolution of CML, nevertheless cannot, by itself, be the sole genetic event giving rise to blast crisis.

Case Summary

The patient was a 42-year-old man who presented with a 1-month history of weight loss, malaise, fatigue, and headaches. His physical exam revealed pallor and splenomegaly without adenopathy. A complete blood cell count (CBC) revealed a hematocrit value (Hct) of 29, platelet count of 32,000, and a WBC count of 430,000 with...
54% blasts, 3% lymphocytes, 4% monocytes, 3% nucleated RBCs, 10% myelocytes, 3% metamyelocytes (metas), 7% bands, and 16% segmented neutrophils (segs). A bone marrow aspirate and biopsy sample revealed a marrow packed with over 90% blasts that were terminal transferase–positive. Karyotypic examination revealed 52 XXY, +5, +11, +20, +3 Ph in virtually all unstained metaphases. In contrast, phytohemagglutinin (PHA)-stimulated metaphases displayed a 46 XY karyotype. Therapy was initiated with oral hydroxyurea followed by two 3-week courses of vincristine, prednisone, and Adriamycin. Six weeks following initiation of therapy, the patient was noted to be in partial remission with an Hct of 28, platelet count of 56,000, and a WBC count of 7,000 with 3% blasts, 8% lymphocytes, 8% monocytes, 5% nucleated RBCs, 11% myelocytes, 12% metas, 15% bands, and 38% segs. The patient refused further chemotherapy and was discharged. Two months later he returned with a peripheral WBC count of 110,000 consisting of greater than 90% lymphoblasts. Therapy with oral hydroxyurea was reinstituted.

Cell Separation

At initial presentation the peripheral blood was diluted 1:1 with plain RPMI, layered over isopytix (Gallard/Schlesinger, Carle Place, NY), and spun at 1,800 rpm for 20 minutes. The interface cells were washed several times in RPMI and upon Wright staining were found to consist of over 85% blasts, with the remaining cells being predominantly myelocytes and metamyelocytes. The RBC-granulocyte isopytix pellet was brought to 25 mL with plain RPMI and mixed with 25 mL 3% dextran (T-500) in phosphate-buffered saline. The RBCs were allowed to sediment for 1 to 1.5 hours, and the supernate was harvested and the cells pelleted. The contaminating RBCs were lysed several times in 0.2% NaCl. The pelleted cells were then washed several times in plain RPMI and consisted of greater than 95% mature granulocytes.

After the chemotherapy-induced partial remission, peripheral blood was subjected to 3% dextran sedimentation followed by lysis of RBCs as described above. These cells consisted of approximately 70% metamyelocytes, bands, and segs, with the remaining cells consisting of mature lymphocytes and monocytes. There were fewer than 5% blasts in this cell population.

At the time of relapse, mononuclear cells were isolated following isopytix gradient centrifugation of peripheral blood. This fraction consisted of greater than 95% blasts.

DNA and RNA Extractions

DNA extractions from the various leukocyte populations were performed by digesting nuclei with protease K followed by phenol/chloroform extraction and ethanol precipitation as previously described. RNA was extracted by homogenizing the cells in guanidine thiocyanate followed by ultracentrifugation through a cesium chloride cushion as described by Chirgwin et al. Poly A–RNA was selected by using oligo-dT cellulose.

Southern, Northern, and Dot Blot Hybridizations

Restriction endonuclease digestion of genomic DNA was performed following manufacturer specifications. Southern and Northern blots were made following standard procedures. DNA dot blot analysis on the total cellular RNA was performed as previously described. All blots were hybridized to nick-translated probes and subsequently washed under exactly the same conditions described previously.

Molecular Probes

5′ abl. A molecular probe from the 5′ end of the c-abl oncogene was cloned from a genomic library constructed from the K-562 CML blast crisis cell line. DNA from this cell line was partially digested with Sau 3A and fractionated by sucrose density gradient centrifugation, and the 15- to 23-kb DNA fragments were isolated. This DNA was ligated with Bam-digested EMBL 3 phage vector DNA, and the ligation mixture was packaged in vitro. Approximately 500,000 recombinant clones were screened with a nick-translated 550-bp HindII-Smal fragment derived from the pABl sub9 plasmid, which contains a v-abl insert. This 550-bp fragment represents the most 5′ sequences of v-abl. Following screening, ten positive clones were isolated and purified. One of these clones contained a 17-kb genomic insert that exhibited a restriction map similar to that of the previously published 5′ end of c-abl, and from this clone a 0.3-kb HindIII-EcoR, fragment free of human repetitive sequences was isolated and used as a molecular probe.

Genomic bcr probe. A 1.4-kb BglII-Sst human genomic fragment representing sequences within the bcr gene that are immediately 5′ to the bcr itself was isolated from a normal genomic library. Details of the cloning procedure will be published elsewhere.

bcr cDNA probes. A 2-kb bcr cDNA clone was isolated from an HL-60 cDNA library screened with the aforementioned 1.4-kb BglII-Sst human genomic bcr fragment. A 700-bp Pst fragment near the 3′ end of this clone that contains sequences that are represented in the normal bcr transcript but not in the aberrant bcr-abl transcript was utilized as a probe designated 3′ bcr.

Jw. A 3.2-kb R-HindIII genomic probe cloned into pBR 322, which encompasses several of the exons within the Jw region of the immunoglobulin heavy chain locus, was obtained from Dr Roger Perlmutter.

v-abl. This probe is a 1.5-kb BglII fragment isolated from the v-abl insert of the pABl sub9 plasmid.

Actin. The actin probe used is a 2.0-kb chick pA 1 β actin cDNA Pst fragment.

RESULTS

Cell Harvesting and Separation

We obtained peripheral blood cells from this patient at three different times during the course of his illness including (1) at initial presentation, at which time he had a WBC count of 430,000 with 54% blasts; (2) during a brief chemotherapy-induced partial "remission" when his peripheral count was 7,000 with >70% terminally differentiating granulocytes and <5% blasts; and (3) during relapse of blast crisis when his peripheral count rose to greater than 100,000 with over 90% blasts. At initial presentation, we separated the immature blast cells from the mature terminally differentiated granulocytes by isopytix gradient centrifugation as described in Materials and Methods. DNA and RNA were extracted from these cells as well as from the mature granulocytes isolated at the time of partial remission and from the immature blasts isolated at the time of relapse.

Lymphoid Nature of the Blast Crisis Cells

Many cases of CML blast crisis are B lymphoid in origin and exhibit immunoglobulin gene rearrangements. This patient's blast cells were positive for terminal transferase, thus suggesting their lymphoid origin. This was confirmed by Southern blot analysis of the blast cell DNA, which revealed several extra nongermline bands hybridizing to a heavy-chain immunoglobulin joining region probe (JH) (Fig 1).
AMPLIFIED bcr-abl GENES IN CML 895

Fig 1. Rearrangement of the immunoglobulin heavy-chain locus in the CML blasts. Genomic DNA was digested with BglII, electrophoresed in 1.0% agarose, transferred to nitrocellulose, and hybridized to a 32P-labeled JH probe specific for the immunoglobulin heavy-chain joining region. Samples include CML blasts isolated at presentation (lane 1); initial presentation granulocytes (lane 2); blasts isolated at relapse (lane 3); HL-60 cells, which exhibit the germline pattern (lane 4); and partial remission CML cells (lane 5). Rearranged fragments in the CML blasts are indicated by arrows.

Fig 2. Enhanced expression of the aberrant 8.2-kb bcr-abl transcript in the CML blasts. Poly A+ RNA (5 μg/line) was electrophoresed in a formaldehyde gel, transferred to nitrocellulose, and hybridized to a v-abl probe. The normal 7.4- and 6.6-kb transcripts as well as the aberrant 8.2-kb transcripts are noted. RNA samples are from K-562 (lane 1), HL-60 cells (lane 2), and the patient CML blasts (lane 3).

RNA Analysis

RNA from the patient’s blast and initial presentation granulocyte populations were subjected to Northern and dot blot analysis. The patient’s blast cells exhibited the abnormal 8.2-kb bcr-abl hybrid transcript that has been previously described in Ph' chromosome-positive CML cells (Fig 2). The relative amount of this transcript is quite high and is comparable with that found in the K-562 cell line, which exhibits a four- to eightfold amplification of the c-abl oncogene (Fig 2). Although we did not obtain enough granulocyte RNA to perform Northern blots, we did note by RNA dot blot analysis a marked decrease of at least eightfold in the level of abl-related transcripts in the granulocyte population compared with the blasts (Fig 3). In addition, a similar eightfold decrease in the level of bcr-related transcripts was noted in the granulocyte population v the blasts. These results indicate that the level of bcr-abl hybrid transcripts is significantly higher in the blasts v the granulocyte population in this patient. It is of interest that hybridization of this dot blot to a 3' bcr probe, which measures levels of bcr transcripts from normal chromosome 22 but does not detect bcr-abl hybrid transcripts, reveals a similar decrease in levels of normal bcr transcripts in the granulocyte populations v the blasts (Fig 3). As a control, we hybridized these same samples to an actin probe that revealed similar levels of actin-related transcripts in both (Fig 3).

c-abl Rearrangement and Amplification in Both Blasts and Granulocytes

We hybridized Southern blots of restriction digests of DNA from patient blasts and initial presentation granulocyte fractions with a 300-bp R1-HindIII c-abl probe (probe
B) located within a 12-kb *BglII* fragment on chromosome 9 at the 5' end of *c-abl* (Fig 4). This probe detected an extra fragment in both the blast and granulocyte DNA digests, thus indicating that the break on chromosome 9 in this patient lies within this 12-kb *BglII* fragment and that both the blast and granulocyte subpopulations harbor a similar chromosome 9 breakpoint (Fig 4). In both the blast and granulocyte fractions, this probe hybridizes more intensely to the rearranged *BglII* fragment than to the germline fragment, which suggests that the rearranged *c-abl* fragment is amplified in this patient. We also noted a rearranged and amplified *KpnI* fragment in genomic digests of blast and granulocyte DNA hybridized to a chromosome 22 probe (probe A) located just 5' to the breakpoint cluster region on chromosome 22 (Fig 4).

**Fig 4.** Amplification and rearrangement of *abl* and *bcr* sequences in CML blasts and granulocytes. Genetic maps of the *bcr* region on chromosome 22 (A), the 5' *c-abl* region on chromosome 9 (B) and the 9:22 *Ph* breakpoint region on the *Ph* chromosome in this particular patient (C) are shown. These maps were derived from data from references 4, 5, and 17 and from our own mapping data. The approximate breakpoints on chromosomes 22 and 9 in this patient have been previously mapped and are indicated by arrows. Probe A is a 1.4-kb *BglII*-Sts chromosome 22 fragment located immediately 5' to the breakpoint cluster region (BCR) and hybridizes to a 19-kb *Kpn* germline fragment as indicated in A. Probe B is a 0.3-kb R-HindIII fragment located in the 5' *c-abl* region of chromosome 22 that hybridizes to a 7.8-kb *BglII* germline fragment as indicated in B. *c-abl* exons are denoted by **. These probes were hybridized to Southern blots of genomic digests of CML DNA from patient blasts (lanes 1 and 3) and from initial presentation patient granulocytes (lanes 2 and 4). Lanes 1 and 2 are *BglII* digests; lanes 3 and 4 are *Kpn* digests. In both blasts and granulocytes probe B identifies the 12-kb germline *BglII* fragment as well as a rearranged, amplified 7.5-kb fragment as indicated (lanes 1 and 2). Probe A identifies the 19-kb *Kpn* germline fragment as well as a rearranged, amplified 7.8-kb fragment as indicated (lanes 3 and 4). These rearranged fragments are 9:22 junction fragments as indicated in C. Densitometric scanning reveals a ratio of rearranged:germline fragment of approximately 6:1 using probe B (lanes 1 and 2) and 10:1 using probe A (lanes 3 and 4) in both the blast and granulocyte DNA digests.
 различными генами при бластной кризисе.

Выводы

1. В клетках бластного кризиса, как и в терминально дифференцированных гранулоцитах, отмечена большая степень экспрессии гена c-abl, что позволяет использовать этот показатель для диагностики и следования за состоянием пациента.

2. Уровень экспрессии гена c-abl может использоваться в качестве маркера при диагностике и мониторинге терапии.

3. Экспрессия гена c-abl позволяет выделить два типа гранулоцитов: бластные и дифференцированные, что имеет большое значение для понимания динамики заболевания.

4. Изучение генетической структуры гранулоцитов позволяет более точно определить стадию заболевания и прогнозировать дальнейшее развитие.

5. Использование методов геномной гибридизации и сканерной денситометрии позволяет более точно определить генетические изменения в клетках, что может помочь в раннем выявлении возникновения бластного кризиса.

6. Изучение генетических особенностей гранулоцитов может помочь в разработке новых методов терапии и прогнозирования исхода заболевания.

7. Все эти исследования показывают, что генетические изменения играют важную роль в развитии бластного кризиса и что необходимы дальнейшие исследования для полной разработки методов диагностики и лечения этого заболевания.
exactly proportional to the decrease in the level of Ph\(^1\) chromosome \(bcr-abl\)-related transcripts in these cells (Fig 3). Indeed, one hypothesis to explain the progression of CML to blast crisis is that a subset of Ph\(^1\)-positive CML cells harboring trans-activating factors specific for the normal \(bcr\) gene might also develop enhanced expression of the aberrant \(bcr-abl\) transcript and presumably gain a selective growth advantage over other Ph\(^1\)-positive CML cells that do not harbor such specific factors. This model would explain how some CML cell populations (for instance, the granulocytes from this patient) would not display high levels of the \(bcr-abl\) transcript even though they harbor multiple Ph\(^1\) chromosomes. An alternative hypothesis to explain the evolution of CML is that a further genetic event or mutation, perhaps involving another oncogene in addition to the Ph\(^1\) translocation, gives rise to blast crisis.

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